Analysis of cellular gene expression changes associated with hepatitis B virus infection and hepatocellular carcinoma

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Abstract

Hepatitis B virus (HBV) replication involves virus-host cell interactions. The identification of host cell factors involved in HBV infection can enhance the understanding of viral replication, pathogenesis and potential. Transcriptome analysis by RNA sequencing was done with HBV-infected cells to identify gene expression changes due to viral infection. In this thesis, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to validate and analyses selected genes, and potential roles in viral replication. Additionally, cellular genes associated with the development of hepatocellular carcinoma (HCC) were identified as having altered gene expression. Analysis of HBx expression and selected HBx mutants on cellular gene expression revealed associations with HCC.

Table of Contents

List of Tables

List of Figures

Introduction

Basic biology and replication of hepatitis B virus

Hepatitis B virus (HBV) is a hepatotropic DNA virus that causes an acute and chronic infection. Perinatal and early infants' infection of HBV increases the risk of developing chronic hepatitis, which remains a major health burden worldwide (1, 2). Without effective therapeutic intervention, 25-40% of the chronic HBV carriers can develop liver fibrosis, cirrhosis, and even hepatocellular carcinoma (2). The World Health Organization estimates that in 2015, 257 million people were chronically infected with HBV, and 887,000 of them died from HBV-related liver diseases, despite widespread HBV immunization (178). Currently approved therapeutics, including interferon- α (IFN- α) and nucleoside reverse transcriptase inhibitors (NRTIs), can effectively suppress viral replication. However, they cannot completely clear the infection. Furthermore, their therapeutic potentials are limited by subsequent adverse effects and emerging RT mutations that confer drug resistance due to prolonged use (3-5). Therefore, alternative therapeutic options are desperately needed to address this global health burden.

The infectious HBV virion is 42 nm in diameter and comprises an outer envelope of surface antigens (HBsAg), an inner nucleocapsid core, a virally encoded polymerase, and a partially double-stranded viral genome of 3.2 kb (6-8). It is the smallest DNA virus with a genome that encodes four open reading frames (ORF) that are partially or wholly overlapping (8). Upon infection, the viral life cycle of HBV is heavily dependent on the interaction between the virion and

cellular host factors, from entry to secretion. Several cellular factors that interact with the virus are revealed to play critical roles in the HBV life cycle and become the targets for antiviral purposes (9). For example, Yan et al. identified sodium taurocholate co-transporting peptides (NTCP) (10), a bile acid transporter expressed in the liver as the receptor for HBV and HDV. It is encoded by the host *SLC10A1* gene and typically regulates the uptake of bile salts into hepatocytes (11). Upon HBV infection, the N-terminal myristoylated PreS1-region of L-protein of HBV binds to the NTCP receptor on the sinusoidal plasma membrane in high affinity to initiate the viral entry (10, 12, 13). The discovery of this virus-host interaction leads to the development of many host-targeting agents, including myrcludex B (an HBV preS1-derived synthetic lipopoly-peptide that binds to NTCP and block HBV infection), cyclosporin A (CsA) (an immunosuppressant that blocks the transporter activity of NTCP), and R041-5253 (a selective antagonist of the retinoic acid receptor (RAR) with the ability to downregulate the expression levels of NTCP to reduce the host susceptibility to HBV infection) (12, 14, 15). A major challenge that hinders the development of HBV antivirals is the unclear characterization of cellular factors and their functions in the viral life cycle, especially at the process of uncoating and synthesis of covalently closed circular DNA (cccDNA) that constitutes the template for all viral mRNA (9). Therefore, the search of host factors that interact with HBV can provide insight into a complete viral life cycle mediated by viral-host interactions and further help identify potential antiviral targets.

RNA Sequencing (RNA-Seq) is a high-throughput sequencing-based approach that maps and quantifies transcriptomes to assess the overall functions and structure of genomes for interpreting the molecular mechanism underlying pathological conditions. A typical RNA-Seq experiment starts from converting the long RNAs to a library of cDNA attached to adaptors. The sequencing of each cDNA using high-throughput sequencing technology generates short sequence reads with sizes 30-400 bp. These resulting reads are aligned with the reference genome or transcriptome to produce a genome-scale transcriptional map that provides information regarding the structure and expression level of each gene (16). The functions and pathways of transcripts from RNA-Seq are analyzed through Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (17). The combination of RNA-Seq and other bioinformatics tools has been widely employed to identify potential cellular factors involved in viral infection and cancer development. In addition, it also reveals the role of host factors during pathogen infection to improve the understanding of viral-host interaction underlying pathogenesis (17-19). Yumeng McDaniel, a Ph.D. student in the lab of Dr. Louis Mansky, has previously conducted a transcriptome analysis of cellular gene expression changes induced by HBV infection. In that analysis, 220 genes were identified having altered expression, of which 131 were protein-coding genes. Among those 131 genes, (1) 14 genes were found to be previously reported to be related to HBV infection; (2) about 23 genes were found to be associated with other viral infections; (3) 13 of those 131 genes were noticed to

be associated with HCC development. The genes that had significantly altered expression upon HBV infection contain substantial potential for diagnosis and therapeutic intervention in HBV-related liver diseases. Therefore, it is necessary to investigate the underlying virus-host cell interactions and the mechanism of altered gene expression in HBV-infected cells.

The life cycle of HBV heavily relies on the interplay between the host and viral functions (9). HBV infection starts from the attachment of the antigenic loop (AGL) in the S domain of all surface antigens to the low-affinity HBV receptor, heparan sulfate proteoglycans (HSPG) on hepatocyte membrane (20, 21). Recently, Verrier et al. identified glypican 5 (GPC5) is another critical receptor for the initial attachment of HBV and HDV at the surface of hepatocytes (22). Upon the proteoglycan binding, HBV entry is processed by a more specific and higher affinity interaction of the myristoylated N-terminal polypeptide in the PreS1 domain of the large surface antigen (L-HBsAg) and sodium taurocholate co-transporting peptide (NTCP) on hepatocytes (10, 12, 13). The receptor binding is followed by the clathrin-mediated endocytosis regulated by the formed complex of L-HBsAg, clathrin, and its adaptor protein AP2 (23). Following viral internalization, HBV utilizes the Rab5 and Rab7-dependent transport through the endosome compartment to proceed viral uncoating (24). Nevertheless, information regarding the host factors and their functions during the de-envelopment and nucleocapsid transport to the nucleus is limited (8, 9). It has been reported that the translocation of capsid to the nucleus requires the binding of nuclear localization signal (NLS) in the COOH-terminal of core proteins and

the nuclear pore complex (NPC) of the nucleus mediated by the nuclear transport factors importins (karyopherins) α and β (25). In addition, a protein of the nuclear basket, nucleoporin 153 (Nup153), is revealed to interact with the nucleocapsid for capsid disassembly and release of the viral genome - polymerase complex into the nucleoplasm after the binding of core and NPC (26).

The partial double-stranded genome, which is also called the relaxed circular (rc) DNA, comprises a complete minus strand (complimentary to the RNA) that covalently attached to the polymerase at its 5' terminus and contains a terminal redundant sequence and an incomplete synthesized plus-strand with a covalently attached RNA oligonucleotide at its 5' end (27). It is converted to the stable genomic template, covalently-closed circular (ccc) DNA for transcription of all progeny genomes. The conversion of rcDNA to cccDNA requires the removal of the polymerase, RNA primers on the 5' terminus of the minus and plus-strand, and the filling and ligation of the gaps (28). Although the exact mechanism regarding cccDNA formation remains largely unknown, many studies speculate it mainly depends on the catalyzation of the cellular DNA repair machinery (27-29). Königer et al. reported that tyrosyl-DNA-phosphodiesterase (TDP) 2 can specifically cleave the tyrosyl-DNA phosphodiester bond that links the viral polymerase and rcDNA to release pol in the process of cccDNA formation (30). Another critical step of the conversion is the "filling the gap" or completion of the plus-strand synthesis of rcDNA. Previous studies examining the contribution of the viral polymerase in cccDNA formation of different hepadnaviruses uncovered that the selective inhibitor of the viral polymerase did not block the conversion,

implicating that cellular polymerase plays an important role (31-33). Recently, DNA polymerase κ encoded by POLK is reported to be essential for the cccDNA formation during *de novo* HBV infection in cell cultures. It is speculated that this cellular polymerase primarily contributes to the formation of cccDNA by filling in the gaps on the plus-strand of the rcDNA (34). Additionally, Flap endonuclease1 (FEN1) is responsible for the removal of RNA or DNA primers during DNA synthesis at the lagging-strand and in DNA repair is involved in the cccDNA formation, presumably by cleaving the 5'-flap structure produced in the rcDNA-specific structure (35, 36). DNA ligase 1 (LIG1) and 3 (LIG3) are required to seal breaks of rcDNA at the final ligation step of cccDNA formation (37). Double-strand linear DNA (dslDNA) is a minor product of DNA replication that can convert to cccDNA via host cellular non-homologous end-joining pathways (NHEJ) and contribute to the integration into host chromosomes, which is a major mechanism of proto-oncogenesis of hepatocytes (38, 39). Ku80 and ligase 4 (LIG4) are two components of the NHEJ pathway essential for converting dslDNA to cccDNA (37, 39).

The cccDNA is organized into nucleosomal complexes with histone and non-histone proteins in the nucleus of the infected hepatocytes (40). The minichromosome serves as the template for all five viral transcripts: (a) 3.5 kb precore mRNA, (b) 3.5 kb pre-genomic (pg), (c) 2.4 kb LHBsAg, (d) 2.1 kb MHBs and SHBsAg, and (e) 0.7 kb transcripts encodes X (8, 9).

The transcription activity of the supercoiled cccDNA is regulated by the recruitment of various proteins, including epigenetic factors, transcriptional

factors, chromatin-modifying enzymes, histone acetyltransferases, and deacetylase onto cccDNA. While the overexpression of histone acetyltransferases such as p300, CREB binding protein (CBP), p300/ (CREB binding protein) associated factor (PCAF) enhances HBV replication, the presence of deacetylases including histone deacetylase 1 (HDAC1) and hSirt1 negatively regulate cccDNA transcription (41, 42). Moreover, protein arginine methyltransferase 5 (PRMT5) is identified to trigger a symmetric dimethylation of arginine 3 on H4 (H4R3me2s) on cccDNA to repress cccDNA transcription (43). The transcription of viral transcripts and HBV integrants is mediated by the host RNA pol II (44). The synthesized transcripts are then exported to the cytoplasm by the interaction between the core protein and the cellular factor Tip-associated protein/ nuclear export factor-1 (TAP/NXF1) for subsequent RNA translation, pgRNA encapsidation, and reverse transcription (45, 46). The five viral transcripts are translated to seven HBV proteins using the cellular translation machinery. The precore RNA encodes the precore protein, converted to the hepatitis B e-antigen (HBeAg) by the host furin endopeptidase (47). The bicistronic transcript, pgRNA, is responsible for producing the core (21 kD) and polymerase (90 kD) proteins. Moreover, it serves as the template for reverse transcription during HBV replication. Its transcript is even longer than the genome length, since it has a terminal redundancy of 120 nt that includes a second copy of the direct repeat (DR1) and the encapsidation signal ε, a stem-loop structure folded at 5’ end that facilitates the process of encapsidation, activation of polymerase, and reverse transcription (8, 48). The core subunits dimerize and

assemble for the formation of the nucleocapsid (49). The C-terminal of core proteins contains serine phosphorylation sites that regulate HBV viral replication and maturation (50).

The reported host cell kinases that mediate the phosphorylation process include cdc2-like kinases, cyclin-dependent kinase 2 (CDK2), Polo-like-kinase 1 (PLK-1), Protein kinase C- α (PKC-α), SR protein-specific kinase 1 (SRPK1), and SRPK2 (51-55). Chen et al. reported that SRPK1 is a host kinase chaperone hijacked by HBV to regulate the core protein assembling and RNA-binding (56). PKC -α inhibition induces accumulation of intracellular capsid and reduced encapsidation capacity (57). The complex functions of other host kinases in the process of capsid assembly remain to be characterized. As the core subunits dimerize, the molecular chaperone heat shock protein 90 (Hsp90) binds to the core dimers. Hsp90 facilitates capsid formation, while another molecular chaperone, Hsp40, negatively regulates viral replication by enhancing the degradation of the viral core and HBx protein (58, 59). The polymerase binds to the encapsidation signal ε, and their interaction triggers the incorporation of the viral RNA and the pol into the capsid for nucleocapsid assembly (60). Host factors that assist their interaction include eukaryotic translation initiation factor 4E (eIF4E), Hsp90, and other cofactors (61, 62). The encapsidated pgRNA interacts with the tyrosine (Tyr) residues in the TP domain, RT domain, and RNase-H domain of pol to synthesize the minus-strand DNA, degradation of pgRNA, and the synthesis of the incomplete plus-strand DNA (63).The nucleocapsid containing the synthesized rcDNA can choose to be transported

back to the nucleus to replenish the cccDNA pool or be enveloped with the translated surface antigens and released as infectious particles.

HBV surface antigens are translated from the 2.4 kb L-HBsAg and 2.1 kb MHBs and S-HBsAg transcripts. The 2.4 kb mRNA transcript encodes the largest surface antigen, 42 kD L-HBsAg, which constitutes Pre-S1, Pre-S2, and S regions. In comparison, the 2.1 kb mRNA transcript expresses a middle-sized 33 kD M-HBsAg that contains the Pre-S2 and S region, and the smallest 26 kD S-HBsAg only with S region (8, 9). Once the surface polypeptides are translated, they are post-translationally modulated by N-glycosylation and transported to the membrane of the endoplasmic reticulum (ER) by the topogenic signals of the S region (64). M-HBsAg, however, contains an additional N-linked glycosylation site and an O-glycosylation site at its N-terminus (65). L-HBsAg is modified by N-terminal myristoylation that is critical for viral infectivity (60). The maturation of the M and L-HBsAg requires the folding of the envelope proteins facilitated by the N-glycan processing, Hsp70, and ER-resident proteins BiP and calnexin. Rather, S-HBsAg utilizes the ER luminal protein disulfide isomerase (PDI) for its maturation during the envelope assembly. For viral particle assembly, the synthesized nucleocapsid is recognized and engaged by the NEDD4 E3 ubiquitin protein ligase (NEDD4). Ubiquitinated NEDD4 interacts with γ2-adaptin attached to the L-HBsAg due to the recognition by the ubiquitin-interacting motif of γ2-adaptin. The HBV/NEDD4/ γ2-adaptin-pre-assembly complex is further processed to viral assembled particles in multivesicular bodies (MVBs) by recruiting ubiquitin-binding ESCRT-II complex, ESCRT- III, and Vps4 that

regulate intraluminal budding and membrane fission. MVBs and MVB-derived exosomes fuse with the plasma membrane and released assembled viral particles (66).

The smallest mRNA transcript (0.7 kb) encodes a 154-amino acid protein, X protein (67). It is a multifunctional protein localized in the cytoplasm, nucleus, and mitochondria of the infected hepatocytes (68). In the nucleus, HBx stimulates transcription by its interaction with different transcription factors, including cAMP response element-binding protein (CREB), RNA polymerase binding protein (RBP5), transcriptional factor IIB (TFIIB), transcriptional factor IIH (TFIIH), and nuclear factor kappa B (NF-κB). HBx also modulates the cytoplasmic signaling transduction pathways, including P13K/Akt, PKC, JNK/STAT, and Ras/Raf/MARK via calcium release (69-71). Furthermore, HBx interacts with other host factors relevant to apoptosis, DNA repair, cell proliferation, and epigenetic changes to promote the persistent HBV infection and even the development of hepatocellular carcinoma (HCC) (69, 72). Although the exact contribution of HBx in the replication cycle remains enigmatic, many studies have indicated that HBx enhances the replication efficiency of HBV, both *in vitro* and *in vivo* models. A lower level of the virus was observed in the hepatoblastoma HepG2 cell transfected with the plasmid containing HBx-defective HBV genome (41, 73), but it can be restored to the average level by co-transfection of the plasmid containing the wild-type HBV genome (74, 75). Similar observations were seen in independent animal models. The transgenic mice carrying the X-defective HBV genome generate less virus than the mice with X-intact HBV

genome (76, 77), but crossbreeding of X-negative and positive transgenic mice can rescue the virus titers (77). Similarly, woodchucks receiving an intrahepatic injection of a plasmid carrying WHx-negative HBV genome showed less virus replication to those with wild-type HBV genome (78). These pieces of evidence mutually support that HBx boosts HBV replication.

To further investigate the specific role of HBx in the HBV replication, Belloni et al. targeted the HBV cccDNA transcription step by comparing the cccDNA copies and levels of pgRNA in HepG2 cells transfected with wild type or HBx mutant virus. Only the level of pgRNA was significantly reduced in cells carrying the HBx mutant HBV genome, implying that HBx participates in regulating the cccDNA transcription (41). HBV minichromosome transcription and HBV replication are largely associated with the acetylation status of HBV cccDNA-bounded H3 and H4 histones. During the transcription step, HBx protein regulates the recruitment of acetyltransferase and deacetylase onto cccDNA to influence HBV replication (41, 42). Recently, Kim and Siddiqui reported that HBx recruits methyltransferase onto cccDNA to induce N6-methyladenosine (m^6A) modification of the viral RNA for increasing viral replication. In contrast, m^6A modification mediated by HBx-methyltransferase interaction destabilizes the stability of host RNA, including phosphatase and tensin homolog (PTEN), with well-characterized tumor suppressor activity (79). The association between HBx and hepatocellular carcinoma has been intensively studied since Kim et al. reported that the transgenic mice with the expression of HBx in their liver developed liver tumor (80).

HBV-associated hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the main cancer-related causes of death worldwide. The general 5-year survival rate for liver cancer patients is 3% to 34%, depending on the clinical stages of the tumor diagnosed (179). Chronic hepatitis B and C infection are the major risk factors of HCC and contribute to 80% of HCC cases globally (81, 82). Common mechanisms for HBV-promoted HCC are associated with HBV DNA integration into host genome (83, 84), HBV-encoded oncogenic protein X (69, 71), and accumulated damages in hepatic inflammation (83). Many studies revealed that HBx promotes the development of HCC via several strategies. COOH-terminal truncated (Ct) HBx is the most common integrant that is frequently detected in HCCs (85). Compared with full-length HBx, Ct-HBx can transform immortalized cell lines such as MIHA and HepG2 more efficiently. Sze et al. determined that the increased cell-invasiveness of HepG2 is induced by the activation of C-Jun protein and matrix metallopeptidase 10 (MMP10) by Ct-HBx (85, 86). Furthermore, Ct-HBx transactivates the nuclear factor of activated T cells 2 (NFATC2) to suppress the activity of a critical regulator of glucose metabolism, thioredoxin interacting protein (TXNIP) in hepato-oncogenesis (87). Ct-HBx has a weak pro-carcinogenic role due to the incapability of tumor development in Ct-HBx transgenic mice. Rather, Ct-HBx can enhance diethylnitrosamine (DEN)-induced hepatocellular carcinogenesis in transgenic mice (88). Another relevant pro-carcinogenic activity of HBx in the activation of oncogenic signaling pathways by HBx and its isoforms. HBx can promote the malignant transformation of the

human hepatic cell line L02 via activation of the Notch signaling pathway. The activation of this signaling pathway affects the expression levels of an apoptotic suppressor *Bcl-2*, CDK inhibitor p16, and NF-κB (89). Ser31 phosphorylation of HBx by Akt contributes to hepato-oncogenesis via enhancing the transformation of NIH3T3 cells with activated *ras* oncogene (90). In addition, HBx can activate other oncogenic signaling pathways such as PI3K/Akt-mTOR (91) and Wnt/β-catenin pathway (92) for mediating cell proliferation, regeneration, and apoptosis in HCC development. The interaction of HBx and host factors also provide useful information regarding the pro-oncogenic role of HBx. HBx upregulates the expression of a proto-oncogene *src* substrate, cortactin (CTTN), and their interaction affects CREB1 and its downstream targets, including *Bcl2* and matrix metalloproteinase 9 (MMP9), to modulate cell proliferation, migration, and oncogenic signaling pathways (93). HBx can also cause upregulation of a cellular regulation factor, sirtuin 1 (SIRT1), to promote hepatocarcinogenesis (94). Although these data support a strong correlation between HBx activity and hepato-oncogenesis, a comprehensive mechanism of HBx-promoted HCC remains elusive. Searching for novel HCC-related host factors that interact with HBx may improve the current understanding of the etiology of HBx-related hepato-oncogenesis.

Thesis goals and objectives

Yumeng McDaniel (Ph.D. student, laboratory of Louis Mansky) confirmed gene expression changes of 18 selected genes related to HBV or other viral infections using real-time quantitative PCR (qRT-PCR). Here, the role of viral

factors in regulating the expression of the 18 cellular genes was characterized for a better characterization of host-viral interaction in HBV infected cells. Expression of host genes in the presence of individual HBV protein was quantified by qRT-PCR and compared with mock/vehicle control. In this study, it was found that the individual HBx or polymerase expression did not induce the alteration of those cellular gene expressions observed in HBV-infected cells. HBV capsid downregulated SRFBP1, an essential factor involved in the post-fusion entry process upon HCV infection (95). L-HBsAg and S-HBsAg triggered the downregulation of FBLIM1 (a cell junction protein), NMT1 (an enzyme with Protein-N myristoylation activity), and NPR2 (a receptor with guanylate cyclase activity). However, M-HBsAg was incapable of downregulating these genes. Additionally, using RNA interference assay, we observed the elevated HBV infection rate with the knocking down of SRFBP1, and reduced infection rate with the knocking down of FBLIM1. To explore the mechanism of HBx-promoted hepato-oncogenesis, the effect of HBx on the expression of the 13 HCC-related genes was also investigated. The data showed that individual HBx expression only induced the up-regulation of SSTR5 (a subtype of the somatostatin receptor family) and FLG2 (a protein required for proper cornification) but not the other 10 genes. Moreover, the study of HBx domain(s) critical for viral oncogenesis demonstrated that HBx_F30V and HBx5 mutants differently regulated the expression of four cancer-related genes, HOMER2 (a member of homer family of dendritic proteins), NFYC (a heterotrimeric transcription factor subunit), TP53 (a tumor suppressor), SSTR5 and FLG2. Taken together, the data presented here

offer new insights about HBV-host interaction and HBV-related HCC development, thus contributing to defining novel anti-HBV targets for improving therapeutic outcomes.

Materials and Methods

Cell lines and plasmids

The HepG2 and HepAD38 cell line was a kind gift from Dr. Daniel D. Loeb (University of Wisconsin-Madison, Madison, WI). The HepG2-NTCP cell line was graciously provided by Dr. Haitao Guo (Indiana University, Indianapolis, IN).

The plasmid pHAGE mNeonGreen-Core (HBc from HBV) IRES puro was a gift from Raphael Gaudin (Addgene plasmid # 122202) (96). HBx, pol, S-HBsAg, M-HBsAg and L-HBsAg were gifts from Wang-Shick Ryu. (Addgene plasmid # 65463; 65520; 103013; 103012; 103011) (97-99). pGFP-HBx was a gift from Xin-Wang. (Addgene plasmid # 24931) (100).

The plasmids pCMV-deltaX and pHAGE-mNeonGreen-deltaCore were modified from HBx and pHAGE mNeonGreen-Core (HBc from HBV) IRES puro, respectively. Briefly, X gene or Core gene were deleted from template plasmids through three rounds of PCR. In the first PCR reaction, linear DNA templates were generated using primers HBx_F0 and HBx_R0, or primers HBc_F0 and HBc_R0 to exclude x or core genes. Using the linear DNA templates generated in the first PCR, fragment pCMV-deltaX1 was produced using primers HBx_Ampicillin_F1 and HBx_blank_R1 during the second PCR, while fragment pCMV-deltaX2 was formed using primers HBx_blank_F2 and HBx_Ampicilin_R2 during the third PCR. Using the linear DNA templates generated in the first PCR, fragment pHAGE-mNeonGreen-deltaCore 1 was produced using primers HBx_Ampicilin_R2 and HBc_blank_R1 during the second PCR, while fragment pHAGE-mNeonGreen-deltaCore2 was formed using primers HBc_blank_F2 and

HBx_Ampicilin_F1 during the third PCR. For the last step, pCMV-deltaX1 and 2 fragments were ligated using Gibson Assembly Master Mix (New England BioLabs Inc.). So were pHAGE-mNeonGreen-deltaCore 1 and 2 fragments. Ligation products were transformed into E. coli, and vectors were purified (Monarch ® Plasmid Miniprep Kit, New England BioLabs Inc) and sequenced using CMV-F or HBc blank-Sanger primer. Sequence alignments were performed using the SeqMan assembler of the Lasergene 7 software package (DNAStar, Madison, WI). The plasmid pcDNA3.1+C-HA was purchased from GenScript and the HA tag was deleted.

The HBx5 and HBx_F30V mutants were generated from HBx plasmid through site-directed mutagenesis using the Gibson assembly approach. DNA fragments 1 and 2 of both mutants were prepared with appropriate site-directed mutagenesis primers in two rounds of PCR. The ligation of fragments 1 and 2 of each mutant was carried out by Gibson Assembly Master Mix (New England BioLabs Inc.) according to the manufacturer's protocol. The ligation products were transformed into *E. coli*, and mutants were purified (Monarch ® Plasmid Miniprep Kit, New England BioLabs Inc.) and sequenced using HBx_Sanger_F primer. Both mutant clones were verified through sequence alignments on the SeqMan assembler of the Lasergene 7 software package (DNAStar, Madison, WI). Primers used are listed in **Table 1**.

Cell Culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal clone 3 (FC3) serum (HyClone, Logan, UT), 100

U/ml penicillin, 100 ug/ml streptomycin, and 100 U/ml MEM-NEAA (Life Technologies). HepAD38 cells were maintained in RPMI-1640 with 10% FC3 serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 U/ml MEM-NEAA 10 mM HEPES, 0.4 mg/ml G418 and 0.3 µg/ml tetracycline. HepG2-NTCP cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% FC3, 100 U/ml penicillin, 100 µg/ml streptomycin and 8 µg/ml blasticidin (101). All cell lines were maintained at 37 °C in 5% CO2.

Production of virus stocks

The HepAD38 cell line produces HBV particles under the control of a tetracycline operator/CMV promoter (102) Five days following removal of tetracycline, cell culture supernatants were collected every other day till Day 29, pool together, and then concentrated to 70-100X by using a Vivaspin 20 ml 100,000 MWCO (Sartorius) centrifuged at 2000 x g at 16°C. The virus was then aliquoted and stored at -80°C until use.

Virus infection

HepG2-NTCP cells were incubated with HBV stock in the presence of 4% PEG8000 and 2% dimethyl sulfoxide (DMSO), centrifuged at 1000g for two hours, and then put in a 37 C CO2 incubator immediately. After 24 h, cells were washed with PBS once and media was replaced with fresh media containing 2% DMSO.

RNAseq

The infected HepG2-NTCP cells were harvested three days post-infection. RNA was extracted from HepG2-NTCP cells with the High Pure RNA Isolation Kit

(Roche) and used for RNAseq. All samples passed initial QC, and NGS sequencing libraries were prepared using the Illumina Truseq library prep kit. All libraries passed QC and were sequenced up to 40-50M reads. Once obtained, FASTQ files were trimmed to remove adapter sequences. FastQ files were then converted to a SAM format and aligned using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts). HTseq was then used to obtain copies per million (CPM) values. CPM is the number of reads for a given gene relative to the total number of reads/million in a sample. For example, if gene A had 100 counts and there were a total of 50M reads then the CPM would be 100/50=2. The CPM data was then used for the analysis as described below. To determine gene expression, the DESeq2 method was used. Here, the CPM for the control samples were normalized to the mean total CPM for all genes for all control samples. The CPM for the treated samples were normalized to the mean CPM for all genes for all treated samples. These data were then filtered to remove genes where there were no copies detected (CPM=0) in any of the 6 samples. A second filter was applied to remove bias by removing samples where <2 control or treated samples gave CPM=0. Control and test samples were then compared and analyzed to determine statistically significant changes in CPM. Principal component analysis was performed to determine clustering between sample groups.

pGFP-HBx transfection and flow cytometry analysis

HepG2 and HepG2-NTCP cells were grown in 24-well plates with a cell density of 150K/well or 200K/well one day prior to transfection. Both cells were

transfected with pGFP-HBx using Lipofectamine 3000 (Life Technologies). Media was replaced one day after the transfection. Four days post-transfection, cells were detached by trypsin EDTA and transferred to a 96-well plate. Cell pellets were collected after the centrifugation at 500 g for 5 minutes. At last, cells were stained with the staining buffer 1X PBS with 2% FC3 and then analyzed on an LSR II flow cytometer (BD Sciences) after being re-suspended with 2% fetal bovine serum in 1X PBS. Data analysis was performed using FlowJo and GraphPad Prism.

Gel electrophoresis

The purified plasmids expressing individual viral factors, including HBc, HBx, pol, S-HBsAg, M-HBsAg and L-HBsAg were diluted 5-fold with sterile water and added with a 5X loading buffer. The prepared samples and a 1kb molecular weight- size marker was loaded on a 2% agarose gel stained with Ethidium Bromide. The electrophoresis was carried out at the voltage 120 V for 1 hour. Fractionalized bands were visualized under the UV light. The size of individual HBV factors was referred to that of the molecular weight-size marker.

Quantification of viral RNA by qRT-PCR

HepG2-NTCP cells were grown in 24-well plates one day prior to transfection. Then HepG2-NTCP cells were transfected with pHAGE mNeonGreen-Core, HBx, pol, S-HBsAg, M-HBsAg, L-HBsAg using Lipofectamine 3000 (Life Technologies). The transfected HepG2-NTCP cells were harvested three days post-transfection, and their RNA was isolated with the High Pure RNA Isolation Kit (Roche). Next, cDNA synthesis was performed using

the iScript Selected cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The synthesized cDNA was diluted 5-fold and quantified by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad). All Real-Time quantitative PCR was carried out using a Bio-Rad CFX96. The thermal cycling conditions for qRT-PCR were 95°C for 3 minutes, followed by 39 cycles of 5 s at 95°C, and 60°C for 1 minute. Data were normalized to 18s rRNA expression. The used primers are listed in **Table 2**.

Quantification of host RNA by qRT-PCR

Transfection of HepG2-NTCP cells with pHAGE mNeonGreen-Core, pHAGE mNeonGreen-deltaCore, HBx, pCMV-deltaX, pol, S-HBsAg, M-HBsAg, L-HBsAg, pCMV-EGFP, pHAGE-EGFP, pcDNA3.1 (delta HA tag), HBx5, or HBx_F30V was performed using Lipofectamine 3000 (Life Technologies). The expression of the antiviral or HCC associated genes was quantified using iTaq Universal SYBR Green Supermix (Bio-Rad). Data was normalized to mock/vehicle control. The sequence and information of the host gene primer sets are listed in **Table 3**.

Transfection of DsiRNA

The hs.Ri. APOM.13.1, hs.Ri. APOL1.13.1, hs.Ri. SRFBP1.13.1, hs.Ri. FBLIM. 13.1, hs.Ri.CLDN5.13.2, hs. Ri. MAGI2.13.1, hs.Ri. TJP2.13.1 and scrambled negative control DsiRNA were purchased from Integrated DNA technologies (Coralville, IA).

Transfection of HepG2-NTCP with DsiRNAs was performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. 20 nM was the final concentration for DsiRNAs. Transfection efficiency with a TYE 563 Transfection Control DsiRNA (Integrated DNA technologies, Coralville, IA) was 70-100% in these cells. Confirmation of the knock down of CLDN5, MAGI2, APOM, APOL1, SRFBP1 or FLG was confirmed by qRT-PCR.

Statistical Analysis

Statistical analyses were, in part, implemented with GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical significance between groups was analyzed by either a one-sample t-test or by a one-way ANOVA test, as indicated. *P* values that were < 0.05 were concluded to be statistically significant.

Results

RNA sequencing analysis of HBV-infected cells

To identify potential cellular factors associated with HBV infection, my colleague (Yumeng McDaniel, Ph.D. student in the lab of Louis Mansky) had formerly conducted a transcriptome analysis of cellular gene expression changes induced by HBV infection. HepG2-NTCP cells were infected with concentrated HBV virus stock. Total RNA was extracted and subjected to RNA-sequencing analysis. Genes with significant expression alteration were identified by DESeq2 determining significant changes in CPM values for control and treated samples. In the transcriptome analysis, we found that expression of 220 genes is significantly altered in HBV-infected cells (**Appendix Figure 1A**). To further confirm the alteration of 220 gene expression in the control and treated samples, a principal component analysis was performed. Indeed, we observed the significant differences between the cluster groups (**Appendix Figure 1B**). These results suggest that these 220 cellular genes are potentially involved in HBV infection.

Characterization of 220 significantly expressed genes using Gene Ontology Enrichment Analysis

Yumeng McDaniel (Ph.D. student, laboratory of Louis Mansky) characterized the 220 genes with significant expression changes in HBV-infected cells, using Gene Ontology Enrichment Analysis (**Appendix Figure 2**). Through the gene type analysis of these 220 significantly expressed genes, it was observed that 131 genes are protein-coding genes that encode functional

proteins, while the rest of genes either represent unmapped or transcribed into RNA molecules responsible for a variety of functions (**Appendix Figure 2A and Appendix Table 1**). To learn about the structural and functional information of these 131 significantly expressed protein-coding genes, these genes were categorized based upon cellular components and biological processes that they are involved (**Appendix Figure 2B and C**). The products of most of the significantly expressed genes were found to constitute the extracellular region and plasma membrane parts of cells. In addition, it was observed that several biological processes are mediated by the products of these 131 protein-coding genes, especially activities related to response to stimulus, signaling and stress. Based on our observation from these analyses, these 131 protein-coding genes with significant expression changes encode products that construct cellular compartments or macromolecules of cells and regulate critical molecular activities to contribute to HBV infection.

Confirmation of host genes involved in cellular antiviral response and HCC

Previously 131 genes were identified as significantly altered protein-encoding genes in HBV infected cells, and some of these genes had been reported to be associated with virus infection and hepatocellular carcinoma development. Through searching literature relevant to the function and activities of these genes in viral and liver cancer formation, it was found that 42 genes were reported to be related to HBV or other virus infection, and 13 genes were involved in the development of hepatocellular carcinoma (**Appendix Table 2**). Then, the expression alteration of 18 cellular host genes in HBV-infected cells

was validated using qRT-PCR assay (**Appendix Figure 3**). These data indicate that HBV induces the significant expression changes of these 18 cellular genes and 13 HCC-related genes to support HBV infection and HCC development.

Flow cytometric determination of transfection efficiency

Transfection is an important tool that introduces foreign nucleic acids into cultured animal cells for studying the functions of expressed genes and gene products in the cells (103). An effective method to determine transfection efficiency for assessing the expression level of transfected genes and gene products is using a flow cytometer to quantify the expression of the green fluorescence reporter protein (GFP) inserted into the plasmid transfected into cells (104). To determine the transfection efficiency of HepG2 or HepG2-NTCP cells, the plasmid HBx-GFP was transfected into cultured cells and GFP expression level was quantified (**Figure 1**). Results showed that the transfection efficiency of HepG2 cells is approximately 6.2% and that of HepG2-NTCP cells is nearly 8.2%. Similarly, data generated by Yumeng McDaniel resulted in a range of HepG2-NTCP transfection efficiency that was between 5% and 10% (data not shown), suggesting that the transfection rate of HepG2-NTCP cells, without optimization, potentially induces unstable gene expression.

Confirmation of sizes and expression of individual HBV plasmids

The HBV genome encodes seven viral proteins, including three different-size surface antigens, HBV polymerase, core protein, the e antigen and the HBx regulatory protein. Since e antigen is not essential for HBV infection and replication (105) and the size of plasmid expressing HBx has been previously

confirmed (data not shown), we chose plasmids expressing the other five viral proteins (**Figure 2A**). Using 2% agarose gel electrophoresis, the sizes of these plasmids were confirmed (**Figure 2B**). Next, the expression of individual HBV plasmids was validated (**Figure 2C**). The large expression of HBx regulatory protein, core protein, and polymerase was observed in the plasmid transfected cells. In addition, it was found that three domains of HBV surface antigens are expressed in cells transfected with L-HBsAg. Cells transfected with M-HBsAg showed expression of PreS2 and S domains. Lastly, there was only expression of S-domain in S-HBsAg transfected cells. These results match with the reported expression of three envelope protein domains. Altogether, the sizes and expressions of individual HBV plasmids were confirmed.

Examination of individual viral factors regulation on the expression of cellular genes

To determine whether the expression of viral factors causes the alteration of host gene expression, I tested the regulation of viral genes on the expression of 18 significantly expressed genes in HBV infected cells (**Figure 3**). The data showed that the expression of HBx and polymerase do not induce changes of these cellular gene expressions (**Figure 3A and 3C**). However, the HBV capsid significantly downregulates SRFBP1, a cellular factor required for HCV entry (95) (**Figure 3B**). L-HBsAg and S-HBsAg cause the downregulation of FBLIM1 (a cell adhesion protein with filamin-binding activity), NPR2 (the primary receptor for C-type natriuretic peptide), and NMT1(N-myristoyltransferase), but M-HBsAg does not alter the expression of these genes (**Figure 3D and 3E**). In addition, Yumeng

further explored the functions of these significantly expressed genes in HBV-infected cells, using RNA Interference assay (**Appendix Figure 4**). Intriguingly, the data showed the elevated HBV infectivity with the knocking down of SRFBP1, and reduced HBV infection rate with the knocking down of FBLIM1. Thus, based on these results, it was showed that HBV capsid protein induced the downregulation of SRFBP1, and the knocking down of SRFBP1 increased HBV infectivity. Furthermore, FBLIM1 was downregulated by L-HBsAg and S-HBsAg, and HBV infection rate decreased with the knock down of FBLIM1. These observations indicate that SRFBP1 is potentially an antiviral cellular factor interacting with HBV capsid upon HBV infection. In contrast, FBLIM1 could be a critical cellular factor supporting HBV infection, and its expression is majorly regulated by L-HBsAg and S-HBsAg in HBV infected cells.

Exploration of the mechanism of HBx-promoting hepato-oncogenesis

Because HBx had been reported to be associated with the formation of hepatocellular carcinoma (89-94), it is necessary to investigate whether the expression of HBx causes the alteration of the 13 HCC-related gene expression (**Figure 4A**). Surprisingly, wild-type HBx was observed to induce the upregulation of a few HCC-related genes, including TP53 (a tumor suppressor), SSTR5 (a subtype of somatostatin receptor family) and FLG2 (a protein required for proper cornification), relative to the control expression. However, the upregulation of TP53 induced by the wild-type HBx was not different from the effect of the vehicle control, suggesting the upregulation of TP53 was mainly caused by the expression of vehicle control. Several sources have revealed that HBx mutants,

including C-terminal truncated HBx and point mutations heavily contribute toward the development of hepatocellular carcinoma (85, 86, 88, 106). Ma et al. reported that COOH-terminal truncate HBx (*79.3%; n=111*) is very frequently detected in HCC tissues. Similarly, others found that compared with the full-length HBx, Ct-HBx amplifies the downregulation of microRNAs (a class of small single-stranded non-coding RNAs majorly responsible for RNA silencing and gene regulation), increases the invasiveness of the immortalized human liver cell line HepG2, and enhances hepatocellular carcinogenesis induced by a carcinogen to promote tumor development. Additionally, some clinical studies have identified point mutations that show a high association with HCC, including I127T, V131I, F132Y/I/R, V5M/L, and F30V. Combinational mutations such as K130M/V131I double mutation, alternatively recognized as PreS mutation A1762T/G1764A also largely affect the disease progression to hepatocellular carcinoma in HBV-infected patients (107-110). To further explore the mechanism of HBx-promoting HCC at the viral-host interaction level, Huixin Yang (Ph.D. student, laboratory of Louis Mansky) and I planned to examine the regulation of closely-HCC related mutations of HBx on the expression of selected HCC-associated genes (**Figure 4B and 4C**). Our current findings showed that HBx_F30V induces the upregulation of HOMER2 (a member of the homer family of dendritic proteins), TP53, SSTR5 and FLG2, while HBx5 upregulates NFYC (a heterotrimeric transcription factor subunit), SSTR5 and FLG2, suggesting that these two HBx mutants potentially adopt distinct mechanisms to contribute to HCC development. A former analysis had dissected X proteins into 6 domains based

on the homology of HBx from different mammalian hepadnaviruses (180) (**Figure 4B**). Three conserved domains of HBx, A (aa 1-20, 90% homology), C (aa 58-84, 86% homology), and E (aa 120-140, 90% homology) are interspaced by those more divergent domains B (aa 21-57, 60% homology), D (aa 85-119, 40% homology), and F (aa 141-154, 60% homology). To study the effect of a highly conserved mutant to HCC development, random residues on the conserved domains were selected to be mutated (**Figure 4B**) and would be tested whether it causes significant alteration of the expression of these HCC-related genes (data not shown).

Discussion

The discovery of HBV has been one of the most considerable milestones related to public health and medicine in human history. During the 1960s, Krugman and colleagues identified two types of infectious hepatitis (infectious hepatitis (IH) and serum hepatitis (SH)) and distinguished their clinical, epidemiological, and immunological features (111). Also, work by Bloomberg and colleagues described the discovery of the Australia antigen (Au), as they observed the reaction between this antigen produced from an Australian aborigine and an antiserum derived from a hemophilic with transfusion. This antigen was later recognized as serum hepatitis antigen (SH) or hepatitis B surface antigen (HBsAg) and determined to be associated with serum hepatitis, or it is now known as HBV (112-114). Using electron microscopy in the 1970s, Dane and colleagues displayed the infectious virion particles in a 42 nm diameter (115). Starting from the 1970s, information regarding the structures of HBV (especially the viral genome and virion-associated proteins), biological, and clinical implications were revealed for a basic understanding of this long-existing virus since 3rd millennium B.C (116-119). Additionally, a prospective study conducted by Beasley and coworkers in Taiwan established the strong association between the virus and the development of HCC (120). Extensive epidemiological studies confirmed the etiological role of HBV in hepatocarcinogenesis, identifying it as one of the main oncoviruses (121, 122). A major breakthrough of HBV treatments was the introduction of the plasma-derived vaccine with validated efficacy in the early 1980s (123). The

development of subsequent recombinant vaccines expressed in yeast had similarly contributed to the dramatic reduction of the prevalence of HBV and incidence of HCC worldwide, especially in HBV- endemic countries (124). For example, the childhood HBsAg seroprevalence in Taiwan, a highly HBV-endemic area, decreased from 9.8% in 1984 to 0.7%, due to the long-established universal childhood hepatitis B vaccination (125). Correspondingly, the average annual incidence of hepatocellular carcinoma in children 6-14 years also reduced from 0.7 in 1981-1986 (prior to vaccination program) to 0.36 per 100,000 children in 1990-1994 (post-vaccine era), demonstrating the substantial impact of HBV immunization (126).

Another historical landmark of HBV therapy was the development of effective anti-HBV treatment options in the 1990s, including IFN- α and NRTIs. IFN-α has a direct antiviral and immunomodulatory activity to suppress HBV replication noncytopathically in a finite period, while NRTIs only target the reverse transcriptase by acting as chain-terminators in viral DNA synthesis after incorporation into the elongating DNA chain (127, 128). One of the critical endpoints of anti-HBV therapy is HBeAg seroconversion, which represents the loss of HBeAg or the presence of anti-HBeAg in chronic HBV patients (129). Response to standard IFN-α treatment showed a moderate HBeAg seroconversion rate in HBeAg-positive patients. Usually IFN-α-induced response can be sustained after discontinuation of treatments, accompanied by an active immune response against the virus. However, a less durable sustained off-treatment response was observed in HBeAg-negative patients that received IFN-

α (130). Peginterferon (PEG-IFN), the interferon modified with a polyethylene-glycol (PEG) group, is superior to IFN-α in inducing the loss of HBeAg and HBV DNA in HBeAg-positive and negative patients. Furthermore, PEG-IFN treatment maintains a higher sustained response rate relative to IFN-α therapy (129). Lamivudine, the first approved nucleotide analog, showed efficiency of HBeAg seroconversion and HBV DNA suppression, but its therapeutic potential is limited by the high rate of drug resistance mutations (131). Later approved nucleot(s)ide analogues, including entecavir, and tenofovir with low rates of drug resistance demonstrated more antiviral responses, especially suppressing HBV DNA by 6 logs in 1-year treatment compared with lamivudine. Nevertheless, entecavir and tenofovir-induced response displays a low HBeAg seroconversion rate (~20% after 1 year and 40-50% after 5 years), indicating the life-long treatment required for chronic HBV patients (3).

Despite the widespread HBV immunization and antiviral treatment options, the HBV prevalence worldwide only demonstrated a minimal decrease from 4.2% in 1990 to 3.7% in 2005. Moreover, the population infected with chronic HBV even rose from 223 million in 1990 to 240 million in 2005, suggesting the need to develop more effective antiviral therapeutics (132). Currently, the major cellular factors and their functions involved in HBV life cycle have been uncovered, and such information promoted the identification and exploitation of some feasibly druggable targets (7). Nevertheless, more unknown host factors that interact with HBV and its proteins remain to be investigated. Here in this study, we explored the interaction between the single viral proteins and significantly expressed

cellular genes in HBV infected cells and identified some critical host factors with substantial antiviral potentials for enhancing the understanding of HBV-host interactions and enriching the landscape of HBV treatments.

The studies conducted in this thesis of cellular gene expression changes induced by viral factors demonstrated that individual viral proteins, especially HBx and polymerase, led to minimal changes in expression alteration of these significantly expressed genes in HBV-infected cells. This observation indicates that the significant expression changes in these 18 cellular genes were mostly induced by other aspects of HBV expression and/or replication. Yet, a few cellular factors were found that interact with HBV capsid and L-HBsAg, and they may be feasible anti-HBV targets. The first critical host factor is serum response factor binding protein 1 SRFBP1. SRFBP1 is responsible for the regulation of GLUT-4 (insulin-regulated glucose transporter) expression in adipose tissues (133), and cytoskeletal and muscle-specific genes (134, 135). Its overexpression induces changes of cellular morphology and reduction of actin content in cultured cells (135). It is localized in both the cytoplasm and nucleus. In the nucleus, it mainly regulates transcription, while it interacts with G actin in the cytoplasm. Additionally, SRFBP1 was reported as an HCV host entry receptor, recruited to the established HCV receptor CD81 to promote HCV infection. By testing the infectivity level of lentiviral pseudo particles carrying HCV glycoproteins in SRFBP1-silenced cells, Gerald and coworkers determined that SRFBP1 does not affect HCV receptor binding. Instead, it may support fusion uncoating. More evidence supported the role of SRFBP1 in viral coating: SRFBP1 was recruited

to CD81 upon HCV E2 glycoprotein or HCV exposure, and their co-localization was also detected in the Rab5-positive early or late endosome (136). Interestingly, in our study, it was found that the expression of SRFBP1 is downregulated by HBV capsid, a critical regulator of uncoating. Besides, our RNA interference assay implied that SRFBP1 may be a host factor that suppresses HBV infection, contrary to the supporter role in HCV infection. This information indicates that SRFBP1 is a possible host factor associated with the uncoating process in the HBV or HCV life cycle, but it shows different regulatory functions toward HBV or HCV infection.

The regulation of gene expression for these genes could be specific to hepadnavirus, since Gerold and colleagues reported that the SRFBP1 silencing does not affect the infectivity of other viruses such as vesicular stomatitis virus (VSV) or human coronavirus 229E (136). The mechanism of how the regulation of SRFBP1 supports HCV entry remains elusive. However, studies found that HCV modulates the actin-dependent hepatoma spread induced by the antibody engagement of CD81, suggesting a vital role of the cytoskeleton in HCV infection (137). In line with this, SRFBP1 mainly regulates cytoskeletal and muscle-specific genes (135), and interacts with G-actin (136). It is possible that HCV takes advantage of SRFBP1 regulation of the cytoskeleton dynamic for supporting its entry. Similarly, HBV may alter the endogenous mechanism of cytoskeleton-dependent transportation regulated by SRFBP1 to promote its infection and this alteration could be achieved by the interaction between HBV capsid and SRFBP1. Future studies including immunoprecipitation and

colocalization of HBc and SRFBP1, relative expression of SRFBP1-related cytoskeletal genes with the expression of HBc or in HBV-infected cells, and the effect of HBc-SRFBP1 interaction to cellular morphology are necessary to further validate the proposed functions and mechanism of SRFBP1 in HBV life cycle.

Another important host factor identified is filamin binding LIM protein 1 (FBLIM1), also known as migfilin. It is a component of cell-extracellular matrix (ECM) adhesions that contains three structurally distinct regions: A C-terminal region with LIM domains, a filamin-binding N-terminal region, and a central proline-rich region (138, 139). The C-terminal region of migfilin binds to Mig-2, a component of cell-ECM adhesions required for cell shape modulation and was co-localized with Mig-2 at cell-ECM adhesions to regulate cell spreading. The migfilin N-terminal region interacts with the C-terminal domain of filamin to mediate the association of migfilin and actin filaments (139). Filamin is a class of proteins that consist of two 280 kDa subunits, and each subunit contains an actin-binding domain at its N-terminus and 24 repeats β-pleated sheet units (140). The binding of migfilin and filament is critical for actin assembly, as Tu and coworkers observed less filamentous actin in the migfilin-deficient cells (139). Since the dysregulation of cell adhesion could lead to the dissociation of cells and indirectly promotes metastasis, the functions of migfilin in the development of various cancers have been investigated (141). Former studies revealed that FBLIM1 is a multifunctional cell adhesion protein that interacts with different binding partners to either promote or suppress cell migration and invasion (141-144). For example, FBLIM1 promotes GSK-3β-mediated β-catenin degradation

to negatively regulate esophageal cancer cell invasion. (The accumulation of cytoplasmic β-catenin induces the nuclear translocation and initiation of the transcription of specific genes involved in oncogenic transformation (138).) On the other hand, FBLIM1 interacts with epidermal growth factor receptor (EGFR, a critical regulator of cellular processes including proliferation, survival, migration, and differentiation) to contribute to the increased cell migration and invasion in gliomas and oral squamous cell carcinoma cells (142, 143). However, the role of FBLIM1 in viral infections remains to be characterized. In this study, we found that the knock down of FBLIM1 decreased HBV infectivity, suggesting a supportive role of FBLIM1 in HBV infection. Nevertheless, the data presented in this thesis has shown that FBLIM1 is downregulated in HBV-infected cells, and this downregulation could be caused by the expression of L-HBsAg and S-HBsAg. A potential explanation to resolve the controversy between these results could be that FBLIM1 supports HBV infection via posttranslational regulation. Another speculation about the mechanism is that the downregulation of FBLIM1 may further activate other binding factors to increase HBV infectivity.

Immunoprecipitation and co-localization of the L-HBsAg, S-HBsAg, FBLIM1 and FBLIM1-associated binding partners are necessary for enhancing the understanding of the modulation of FBLIM1 to the infection of a typical hepato-tropic virus. Moreover, we observed the downregulation of FBLIM1 induced by L-HBsAg and S-HBsAg, suggesting the PreS1 and S domain of HBV surface antigens mediate the downregulation of this gene. To determine the residues responsible for the expression alteration of these genes, an examination

of L-HBsAg and S-HBsAg mutants' regulation on the expression of FBLIM is necessary to be included in the future studies. The region of FBLIM1 that interacts with the PreS1 and S domain of HBV surface antigens could be also identified using deletion mutants. With more information regarding the interaction between the viral factors and these two important host factors being uncovered, SRFBP1 and FBLIM1 may become potential antiviral targets with improved therapeutic outcomes for HBV patients.

In this thesis, downregulation of NMT1 (N-myristoyltransferase 1) and natriuretic peptide receptor 2 (NPR2) were also observed and were due to the expression of L-HBsAg and S-HBsAg. Detection of the cytosolic and ribosomal fraction, NMT1 catalyzes myristoylation of substrate proteins, allowing them to carry membrane-targeting activities (145). In addition to cellular proteins, many viral proteins are N-myristoylated, including the envelope (E) protein of dengue virus (146), Gag or Nef of HIV-1 (147), L-HBsAg of HBV (148), VP4 of poliovirus (149), VP2 of the simian virus (150), and $pp60^{src}$ of Rous sarcoma virus (151). Myristoylation of viral proteins plays a critical role in regulating viral infectivity (148), replication, and RNA expression (152). The studies conducted as part of this thesis have revealed that NMT1 was upregulated in HBV-infected cells. This result coincides with the posttranslational N-myristoylation of L-HBsAg, which is critical for L-HBsAg to acquire infectivity (60). Similarly, Suwanmanee et al. observed the upregulation of NMT1 in dengue virus-infected dendritic cells at 1-, 12-, and 36-hours post-infection and a decreased viral titer in NMT1-silenced dendritic cells, suggesting that the regulation of NMT1 at different infection

stages, from the membrane-associated viral entry to viral exocytosis facilitates DENV replication (146). Nevertheless, in the study of the NMT1 expression regulated by single HBV viral factors, it was found that the downregulation of NMT1 induced by L-HBsAg and S-HBsAg, contrary to the regulation of HBV.

There is no obvious explanation for the differences between the regulation of the intact HBV and single surface antigens to NMT1 expression, unless more information related to the viral- host interaction and co-localization is uncovered. However, based on the results from current studies, it can be concluded that NMT1 is an essential host factor in the life cycle of HBV and a potential antiviral target for HBV infection. NPR2, a transmembrane receptor with guanylyl cyclase activity upon binding with the ligand C-type natriuretic peptide (CNP) is expressed in chondrocytes and vascular smooth muscle cells (145). NPR2 is one of the human protein kinases (HPKs) that affect multiple host pathways to negatively regulate the replication of Influenza (153), but the mechanism remains inconclusive. Previously our observation showed the upregulation of NPR2 caused by HBV infection, implying that some host pathways modulated by NPR2 are involved in HBV infection. Interestingly, later we found that NPR2 expression was downregulated by L-HBsAg and S-HBsAg. This discovery, again, demonstrates the distinct interaction of this host factor with the complete infectious virion or the single viral factors, leaving the roles of NPR2 and its associated cellular pathways in the HBV life cycle to be further explored.

One unresolved issue that remains to be addressed from this study is the incapability of M-HBsAg in causing the downregulation of these three host

factors. In the early studies of the functions of HBV surface antigens, M-HBsAg was reported that its expression does not affect viral morphogenesis and infectivity (154, 155), leaving the function of preS2 domain inconclusive. Further investigation on the role of the preS2 domain of L-HBsAg was carried out and excluded the possibility of L-HBsAg preS2 region from modulating HBV infection (156). Such evidence may suggest that preS2 domain is dispensable for viral secretion and infection of HBV, while a complete life cycle of HBV requires the interaction with these cellular factors.

Despite extensive studies, the precise role of HBx protein in hepato-oncogenesis is yet to be determined. Due to its pleiotropic functions, HBx may contribute to the development of HCC in several ways, including interfering the DNA repair pathway, regulating apoptosis, epigenetic modification and telomerase activity, and interacting with nuclear transcriptional factors or cellular signaling pathways (71, 157). However, the causal role for HBx in liver carcinoma is not fully supported by current studies because of a few major limitations. The most critical issue in many in vitro HBx experiments is the lack of an authentic model for examining the effect of HBx with low-expression in HBV-associated HCC (157). Furthermore, the pathogenesis of HCC requires long-term liver inflammation caused by the destruction and renewal of liver cells, excluding the strong and direct connection between HBx and HCC (69). Instead, several other groups found the enhanced expression of c-Myc transgene, elevated hepatocytes proliferation, and increased focal lesions associated with the development of HCC, in HBx-transgenic mice that either crossed with c-Myc

oncomice or received treatment of carcinogen, suggesting that HBx could be a weak, assisting supporter in liver oncogenesis (158, 159).

Since the HBx protein is a trans-activator that constantly modulates the transcriptional activity of different transcriptional factors and promoters, its interactions with possible HCC-related host factors are intensively investigated for further deciphering the exact contribution of HBx toward hepato-oncogenesis (69). Based on the findings from former viral-host interaction studies, a few cellular factors such as cortactin (CTTN), and centrosomal P4.1-associated protein (CPAP) were identified to be upregulated by X protein for mediating cell migration and proliferation of HCC or enhancing the activities of HCC-associated signaling transduction cascade (93, 160). However, more unknown HCC-relevant host factors that interact with HBx are yet to be reported. In conjunction with this thesis, it was previously determined that 13 significantly expressed genes are associated with the development of HCC, using the data analysis and literature research of the 220 genes with significant expression changes identified from the RNA-sequencing analysis. Furthermore, the examination of expression changes of these HCC-related genes induced by X protein showed that the full-length HBx could only cause the upregulation of a few HCC-associated genes, including SSTR5, and FLG2.

SSTR5 belongs to the family of somatostatin receptors that are commonly expressed in non-healthy and tumor tissues such as cirrhotic and HCC (161-163). The binding of somatostatin analogues (SSAs) to SSTR, especially SSTR2 and SSTR5 triggers the suppression of cell proliferation to inhibit tumor

development (161). Interestingly, the expression of SSTR5 was reported to be significantly decreased in HCC tissues, compared with the paired surrounding cirrhotic liver cells (161, 162).

While SSTR5 has been identified as an important HCC-associated factor, its interaction with the oncogenic protein HBx is yet to be revealed. This thesis has shown the significant upregulation of SSTR5 was triggered by the expression of HBx, implying that HBx could contribute to HCC development via interacting with HCC-associated factors such as SSTR5. In contrast, in HBV-infected cells, we observed the opposite expression changes of SSTR5, suggesting that this gene could induce antiviral response. This discrepancy between the regulation of the single viral oncoprotein and intact infectious virion on SSTR5 remains to be elucidated, but it has further validated the association of HBV, including HBx with the hepato-oncogenesis. Lastly, the upregulation of FLG2 was also observed and was caused by the expression of HBx. Being a protein involved in the formation of the skin barrier (164), FLG2 was identified and its mutation percentage is related to the size of liver tumor (165). Although the information regarding the functions of FLG2 in HCC development is limited, our observation of the interaction between FLG2 and HBx suggested that dysregulation of FLG2 induced by HBx could promote HCC.

Compared with full-length HBx, C-terminal truncated and point mutants of HBx are more commonly detected from the tissues of HCC patients. The functions of some major HBx mutants in the formation or development of HCC have been reported (71, 110). In this study we examined the expression changes

of selected HCC-associated genes triggered by these major HBx mutants for enhancing our understanding of the mechanism of HBx-promoted hepato-oncogenesis. These results have demonstrated that both the HBx mutants, HBx_F30V and HBx5, could induce the upregulation of more HCC-related genes than the wild-type HBx. In addition, the distinct regulation of these two HBx mutants was observed to different HCC-associated genes. Besides upregulating SSTR5 and FLG2, HBx_F30V also causes the expression changes of TP53 and HOMER2.

TP53 is classified as a tumor suppressor gene responsible for repairing DNA damage and apoptosis to prevent tumor formation (166). Mutations in TP53 are common genetic abnormalities in human cancers, including HCC (167). HBx, the multifunctional regulatory protein, was revealed to suppress a wide range of TP53-related activities, such as p53 sequence-specific DNA binding, p53 transactivation ability, and the interaction of p53 with other cellular factors related to nucleotide excision repair (168). Despite being repressive to p53-associated functions, HBx was reported to controversially regulate the expression of TP53. The expression of p53 gene was significantly repressed by X protein in the hepatoblastoma cell line Huh7 and HepG2-K8 transfected with the full length HBx (169, 170). On the other hand, HBx mediated the upregulation of p53 to elevate the expression of proteasomal activator gamma (PA28γ) for inhibiting the degradation of HBx (171).

In this thesis, however, HBx wildtype did not induce expression change of TP53. Instead, the upregulation of TP53 was observed to be caused by the expression of HBx_F30V. Moreover, the upregulated expression of TP53 by HBx_F30V is contrary to the reported suppression effect of HBx to the p53 gene. This unusual finding might further suggest that mutation on the residue 30 of HBx is critical for the altered expression of TP53. Additionally, this HBx point mutant could adopt distinct pathways to modulate the functions of HCC-associated host factors such as TP53 for the purpose of promoting viral infection and liver tumor development.

HOMER2 is a scaffold protein that regulates intracellular calcium homeostasis. Recently it has been identified as a novel biomarker of HCC and its downregulation is closely associated with the development of HCC (165). Furthermore, the dysregulation of HOMER2 expression was reported to be involved in the tumorigenesis of endometrioid adenocarcinoma (EAC) and rectal carcinoma (RC) (172, 173), also demonstrating that the regulation of HOMER2 on calcium homeostasis has significant impact in tumor development. Here we found the upregulation of HOMER2 induced by the expression of HBx_F30V, displaying an opposite regulation to the finding from the recent study (165). One potential explanation is that the upregulation of HOMER2 in HBV-infected cells helps HBV escape from immune responses, as Luo et al. mentioned that HOMER2 is able to inhibit the activation of T cells (172). On the other hand, the other HBx mutant, HBx5 could upregulate NFYC, SSTR5 and FLG2. NFYC is a subunit of a heteromeric transcriptional factor necessary for binding to the

CCAAT motif in the promoter region of many genes (174). It was reported to assist POU Class 5 Homebox 1 (POU5F1), a transcriptional factor that is associated with various tumors to regulate activities related to tumor development via interacting with other factors, including KAT2A, CCND2 and GPD2 (175). This thesis has shown that NFYC was upregulated by HBx5. Similarly, He et al. observed the upregulation of NFYC in liver hepatocellular carcinoma (LIHC) tissues (175). Taken together, such information might suggest that the NFYC is a critical supporter for tumor development, and its interaction with HBx5 could promote HCC.

The reason that accounts for the divergence between the regulation of the wildtype HBx and mutants on the expression of HCC-related genes requires further investigation. However, there was evidence indicating that the full-length HBx is mainly localized in the cytoplasm (169), instead of the nucleus for transactivation. Rather, some HBx mutants such as double truncation mutants HBx (61-124) were found in the nucleus. The nuclear localization of HBx (61-124) was speculated to be induced by the exposed nuclear localization signal from the conformational changes of that mutant (169). It is possible that the indicated HBx mutants might trigger conformational changes to be localized in the nucleus to interact with more HCC-associated factors to contribute to HCC formation. Nevertheless, this speculation might not be tested until the crystal structure of HBx is revealed (176).

Despite the discovery of some host factors with significant antiviral values, this study still needs a few improvements to be more credential. The first

limitation of this study is the low transfection efficiency of HepG2-NTCP cells, which might lead to the unstable gene expression. According to the report from Nishitsuji et al., the general transfection efficiency of HepG2 cells should be greater than 35% (177). Since the transfection efficiency of HepG2 cells was only 4% in this study, further specific optimizations are required to achieve high transfection efficiencies. Another restriction of this study is the single experimental design. Here, we mainly examined the regulation of HBV viral proteins on HBV and HCC-related host genes via qRT-PCR, due to the time limitations. Without time constraint, the addition of more experiments including immunoblot analysis, immunoprecipitation and co-localization would enhance the credibility and provide more useful information related to the host functions in HBV infection and HCC development.

In summary, the analysis of the regulation of single HBV viral factors to HBV-associated cellular genes was determined and identified host factors with potential therapeutic potential. Additionally, because the exact contribution of HBx to the formation of HCC remains elusive, the expression changes of HCC-related genes induced by the wildtype and mutants of HBx was investigated. The results in this thesis have shown that the full-length HBx is a weak oncoprotein that rarely promote the development of HCC via interacting with host factors commonly expressed in HCC tissues, while HBx mutants can induce the dysregulation of more HCC-associated factors to be involved in the formation and development of HCC. The findings reported in this thesis reveals new information about viral-host interactions that may be important regarding HBV

infection and HCC development, thus enhancing our understanding of a complete HBV life cycle and enriching the landscape of novel anti-HBV targets.

Table 1. Primers used to construct blank control and mutant plasmids

Name	Primer (5'-3')
Hbx_Ampicilin_F1	CCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGG
Hbx_Ampicilin_R2	CCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGG
HBx_F0	GGCTGACTACAAAGACCATGACGG
HBx_R0	CGGTTCACTAAACGAGCTCTGC
HBx_blank_R1	CGGGATCCAATTCTGACGGTTCACTAAACGAGCTCT
HBx_blank_F2	TCAGAATTGGATCCCGGGCTGACTACAAAGACCATG
HBc_F0	CCCCCCCCCCTAACGTTACT
HBc_R0	CAGAACCACCAGAACCTCCC
HBc_blank_R1	AGGGATCCGGAACCACCAGAACCACCAGAACCTC
HBc_blank_F2	GTGGTTCCGGATCCCTCCCCCCCCCCTAACGTTACT
HBc_blank-Sanger	TTCTGGTGGTTCTGGTGGTTCC
Hbx_F30V_F2	GACGACCCGTCTCGGGGTCGCTTG
Hbx_F30V_R1	CAAGCGACCCCGAGACGGGTCGTC
Hbx_Hbx5-G1386A_F2	GGCTGCTAAGCTGTGCTGCCAACTGG
Hbx_Hbx5-G1386A_R1	CCAGTTGGCAGCACAGCTTAGCAGCC
Hbx_Sanger_F	CTCGTTTAGTGAACCGTCAGAA

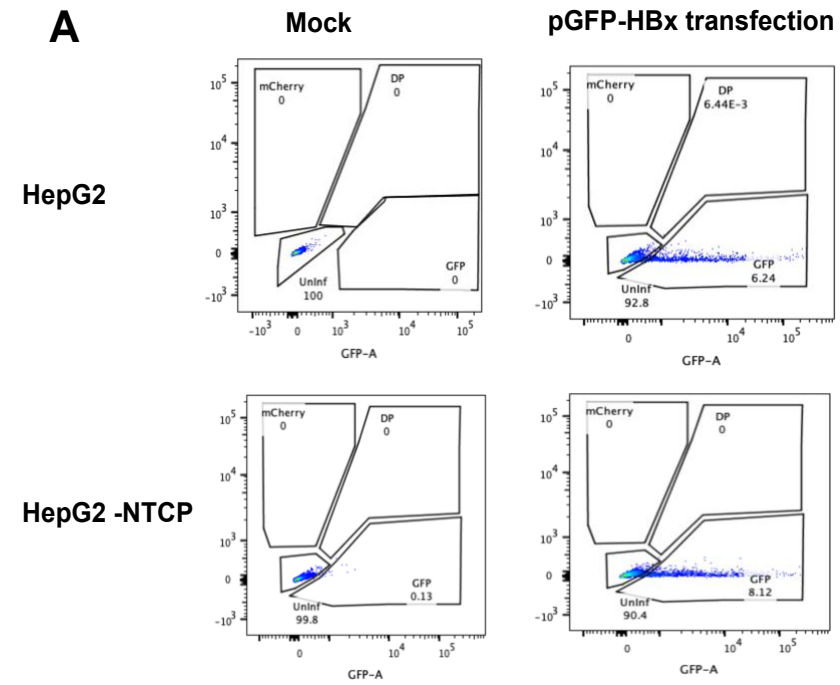
Table 2: Primers used to verify the expression of individual HBV factors

Name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Expected Size (bp)	Reference
18s rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	89	DOI:10.1016/j.antiviral.2017.08.001
HBc	TTCCGGAAACTACTGTTGTTAGAC	ATTGAGATTCCCGAGATTGAGA	98	PMID: 28494793
HBx	CCATCCAATCGGTAGTAGCG	CCCAACTCCTCCCAGTCCTTAA	309	PMID: 10449472
Pol	GCAGGCTTTCACTTTCTCGC	GGTTGCGTCAGCAAACACTT	115	NC_003977.2
PreS region	GGACCCCTGCTCGTGTTACA	GAGAGAAGTCCACCACGAGTCTAGA	89	Yumeng's HBV Paper (Y.M.et al., 2019)
PreS2 region	CCTGTATTTCCCTGCTGGTGG	TTGACGATAAGGGAGAGGCA	77	NC_003977.2
PreS1 region	ACACCGCAAATCCAGATTGG	TTTGTAGTATGCCCTGAGCC	150	NC_003977.2

Table 3: Primers of genes related to antiviral response and HCC

Category	No.	Gene Name	GenBank Accession Nos	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Expected Size (bp)	Reference
HBV Associated	1	APOM	NM_019101.3	CTGACAACTCTGGGCGTGGAT	TGTCCACAGGGTCAAAAGTTGC	118	PMID: 29067439
	2	CTDSP1	NM_021198.3	TGGACAATTCACCTGCCTCC	TGAGCACTGAGTACACGTCG	150	self design
	3	ATP2A1	NM_001286075.2	TCTGTGCCCTCTGCAATGAC	GGCTTCTCACATCCGTGTTG	138	self design
	4	MMP9	NM_004994.3	GTCATCCAGTTTGGTGTCGC	AATGGGCGTCTCCCTGAATG	112	self design
	5	FTL	NM_000164.4	CAGCCTGGTCAATTTGTACCT	GCCAATTCGCGGAAGAAGTG	114	PMID: 26871431
Other Viruses Associated	6	ABCB6	NM_005689.4	CTTCGTCCCCAGTCCTATAC	CCTTCTCAGTCAGCAAGTTC	265	PMID: 17006453
	7	AFMID	NM_001010982.4	ATAGTGGCTTACGGCATCGC	TGCTTGGATACCGCTTCTGG	97	self design
	8	APOL1	NM_001136541.2	CGAGGCATTGGGAAGGACAT	CTGAGGCATGCGGTACTGAC	73	self design
	9	SRFBP1	NM_152546.3	AACATGGACCTAAAGCAGTGAC	GTCAGCAGGTGTCTTCTGGG	98	self design
	10	NMT1	NM_021079.5	GCCATCCCAGCAAACATCCA	GGAGCAACCCTCTTGGAACG	101	self design
	11	NPR2	NM_001378923.1	AACTTGGAGAAGCTGGTGGAGGAA	CTGCTGACAATGCTGTGAAGCCAA	196	PMID: 23805279
	12	SIVA1	NM_021709.3	TGGATGGGAAGGCGGTCTG	ATGTCACTGCAGTCCACGAG	121	self design
	13	DMAP1	NM_001034024.2	GGACGGAGCAATGTTCTTCCA	GAACCTGGCAAAGGGGTAGTC	70	self design
	14	DHX8	NM_001322220.3	ACAGTTCAGAAACGGCAGGA	GTTCGACCTGGGATGGTGAAA	113	self design
	15	FLG	NM_002016.2	CTCTCACCGCGATACAGCC	TGACCATGTTCCTTAGCGGT	78	self design
Cell-cell Adhesion	16	CLDN5	NM_003277.4	GTGCTCTACCTGTTTTGCG	GACGGGTCGTAAAACTCG	86	PMID: 22369552
	17	MAGI2	NM_001301128.2	ATAACAGATCGGCCGCCTCA	GTGGATACGTGCCGTCTAGC	70	self design
	18	FBLIM1	NM_001350151.2	CTGCACCTATGCAGCTCTTC	GGAAGCACGTCCTCACCATC	73	self design
HCC Associated	19	BAD	NM_032989.3	CGAGTCTTCCAGTCCTGGTG	GATGTGGAGCGAAGGTCACT	78	self design
	20	CAB39L	NM_001287338.2	TCAGTCGTCTCATTTGTCCAGT	GCACCTCCAAAGTCTTCCCAA	77	self design

	21	EXOSC9	NM_001034194.2	CAGGCAGTCAGATCTCTTGGT	CCTTTTCACCAGCAACAACACA	107	self design
	22	HOMER2	NM_199330.3	GCTGAAGATTGCCTTGACGC	CATTGCTCTCCCGAAGGGTC	80	self design
	23	HSF1	NM_005526.4	AGTGGCTCAGCACATTCCAT	GATGTCGGAGATGATGGGTCC	159	self design
	24	INO80C	NM_001098817.2	CATTGCCGTGGCAACTGAAC	TGGCAAGCAGACCTGAAACA	102	self design
	25	NFYC	NM_014223.5	TAACAAGCGCCGGACTCTAC	CTCCTGACGCTTTGGAGGTT	121	self design
	26	RFFL	NM_001017368.2	CCTAGACAGTTTCACCCCTCC	ACACATGGGCAAGCATCTCC	72	self design
	27	SMARCA4	NM_001387283.1	ATCCTCACAGGCACAGATGC	CATACCCCGGGTTCATCTCG	70	self design
	28	SSTR5	NM_001172560.2	CCTGACAGTCATGAGCGTGG	CATGCACAGAGACAGGACCC	124	self design
	29	TP53	NM_001126118.2	TTTGAGGTGCGTGTTTGTGC	CCCACGGATCTGAAGGGTGAA	195	self design
	30	FLG2	NM_001014342.3	GGAAAGGTCATGGTGGTCTGT	AACCACATGCATGACTTCGC	144	self design
	31	MED30	NM_001282986.2	ATGTCCACCCCTCCGTTGG	AGCGACGCCGTGTTGACTT	98	self design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Mock
pGFP-HBx transfection
HepG2
HepG2 -NTCP
mCherry
0
DP
0
GFP
0
UnInf
100
GFP-A
mCherry
0
DP
6.44E-3
GFP
6.24
UnInf
92.8
GFP-A
mCherry
0
DP
0
GFP
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UnInf
99.8
GFP-A
mCherry
0
DP
0
GFP
8.12
UnInf
90.4
GFP-A

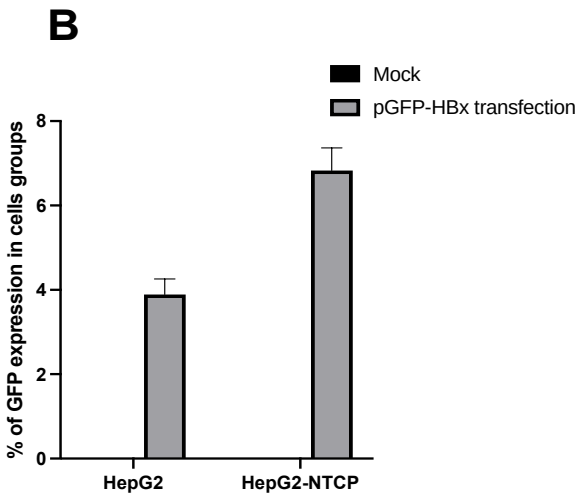
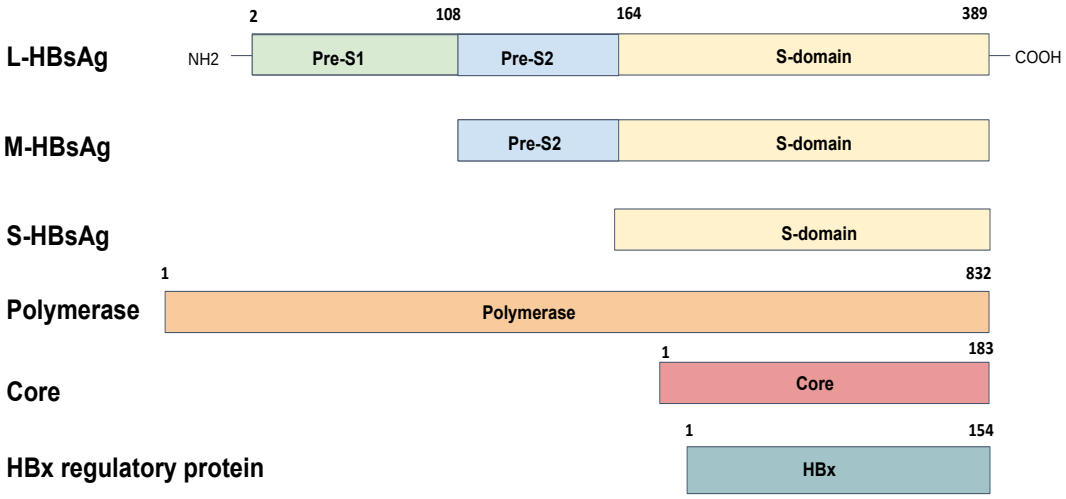

B
Mock
pGFP-HBx transfection
% of GFP expression in cells groups
0
2
4
6
8
HepG2
HepG2-NTCP

Figure 1. Flow cytometric determination of transfection efficiency of HepG2 and HepG2-NTCP cells. A. Flow cytometry analysis. B. Quantitative analysis. HepG2 and HepG2-NTCP cells were transfected with pGFP-HBx. Four days post the transfection, cells were collected and stained with the staining buffer 1X PBS with 2% FC3 for flow cytometry. The amount of GFP fluorescence expression in each transfected cell was determined using an LSR II flow cytometer. Data analysis was performed using Flowjo and GraphPad Prism. Results are shown as average ± standard deviation with two independent experiments.

A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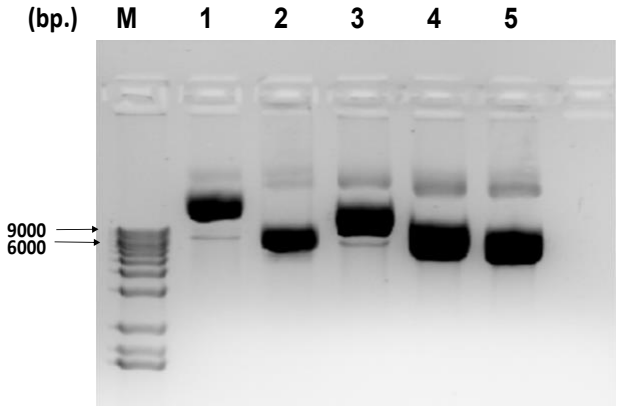
108
164
389
L-HBsAg
NH2
Pre-S1
Pre-S2
S-domain
COOH
M-HBsAg
Pre-S2
S-domain
S-HBsAg
S-domain
1
832
Polymerase
Polymerase
1
183
Core
Core
1
154
HBx regulatory protein
HBx

B


(bp.)
M
1
2
3
4
5
9000
6000

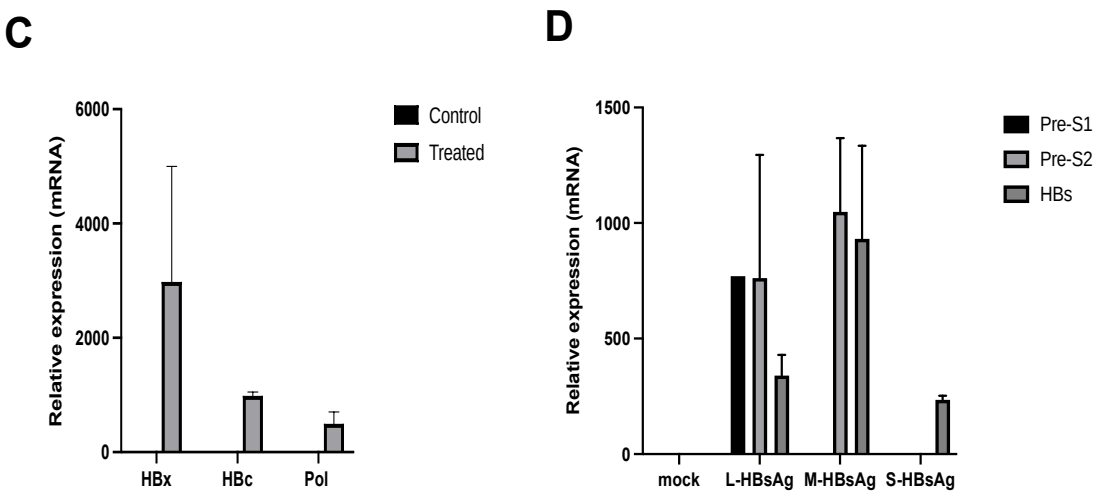
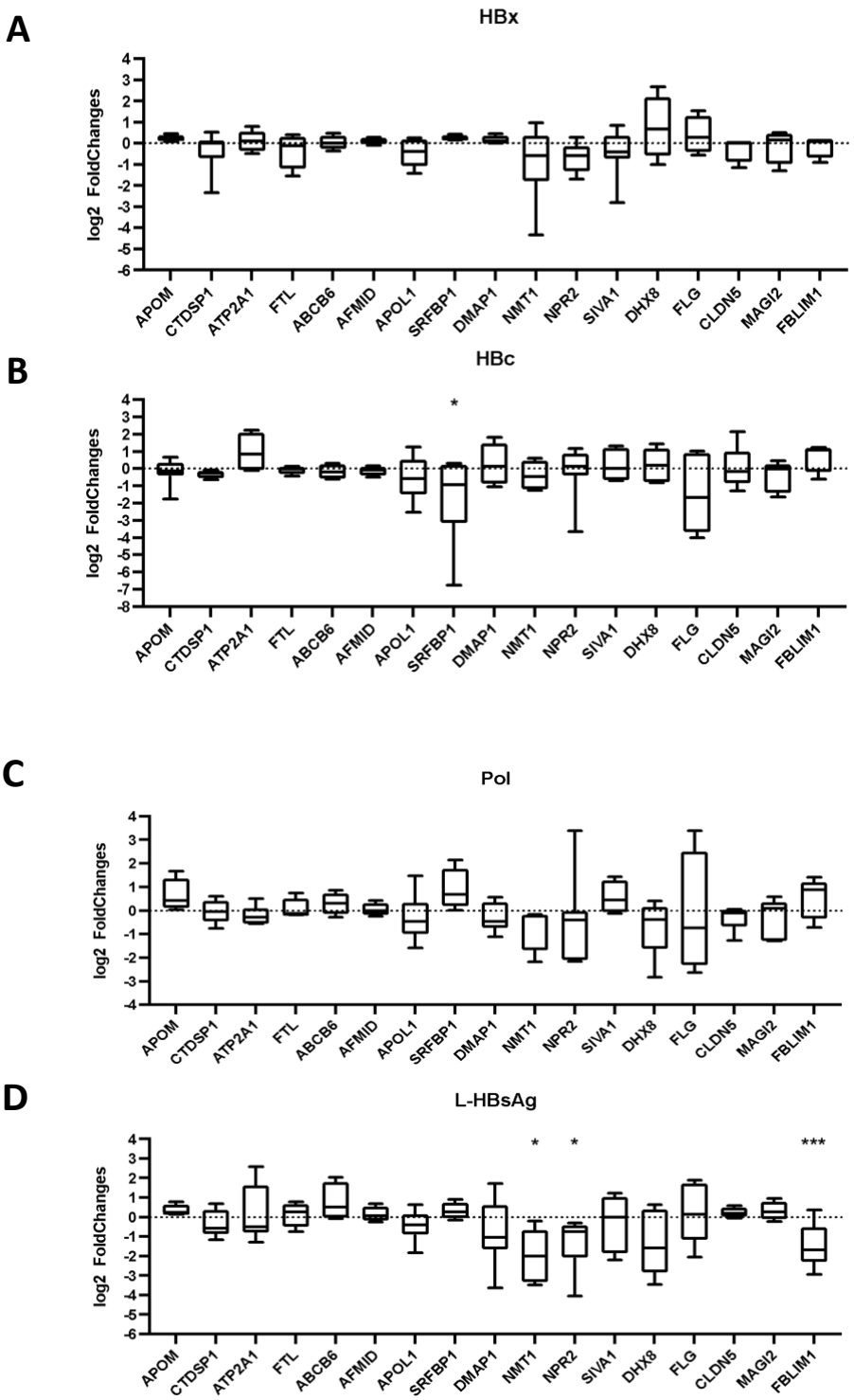


Figure 2. Confirmation of size and expression of individual HBV plasmids. **A. Domain structures of HBV viral factors.** The infectious HBV virion encodes seven viral proteins, including HBV surface proteins, viral polymerase, capsid-forming core proteins, e-antigen and the multifunctional HBV X regulatory protein. Since e-antigen is not essential for HBV infection and replication, it is not shown in this figure. **B. Agarose gel electrophoresis of extracted plasmids that express individual HBV factors.** The size of DNA fragments representing individual HBV factors was referred to that of the molecular-weight size marker. Lanes 1 to 5 represent the following individual viral factors: HBV capsid, M-HBsAg, HBV polymerase, L-HBsAg, and S-HBsAg, while lane M is the 1kb DNA size marker. **C-D. Relative expression of individual viral factors was confirmed via qRT-PCR.** HepG2-NTCP cells were transfected with plasmids expressing HBx, HBV core, HBV pol or L, M, S-HBsAg. After 3 days post-transfection, cells were collected for RNA extraction and qRT-PCR. Data were normalized to 18sRNA expression. Results are shown as average ± standard deviation with two independent experiments.


A
HBx
log2 FoldChanges
APOM
CTDSP1
ATP2A1
FTL
ABCB6
AFMID
APOL1
SRFBP1
DMAP1
NMT1
NPR2
SIVA1
DHX8
FLG
CLDN5
MAGI2
FBLIM1
B
HBc
*
C
Pol
D
L-HBsAg
* *
***

E

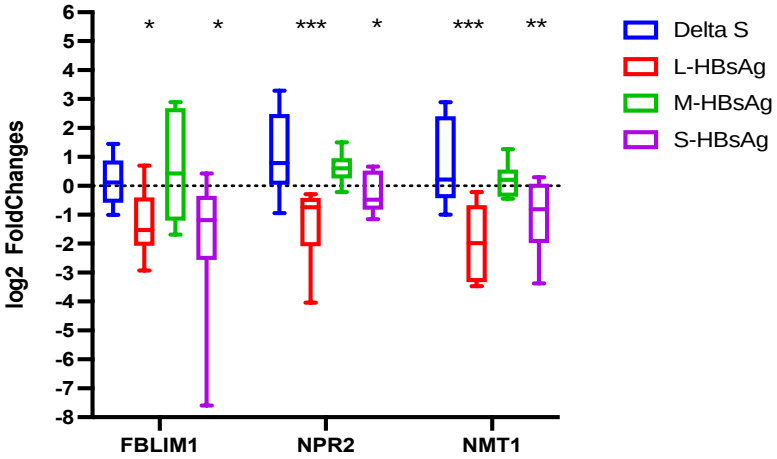
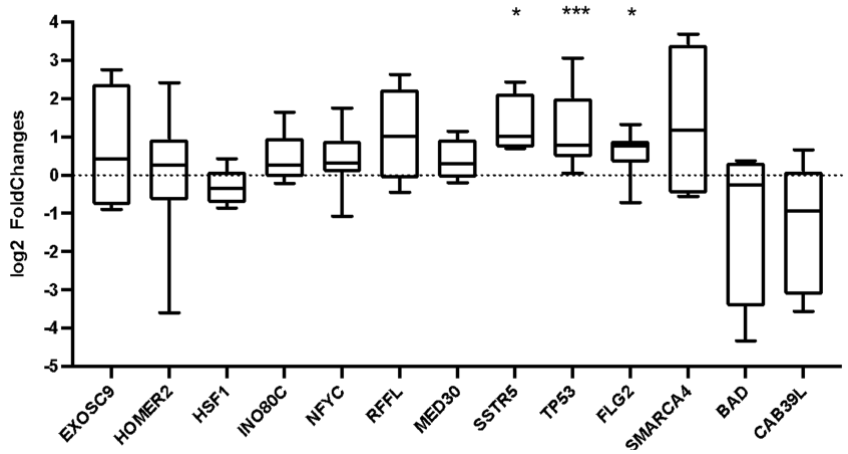
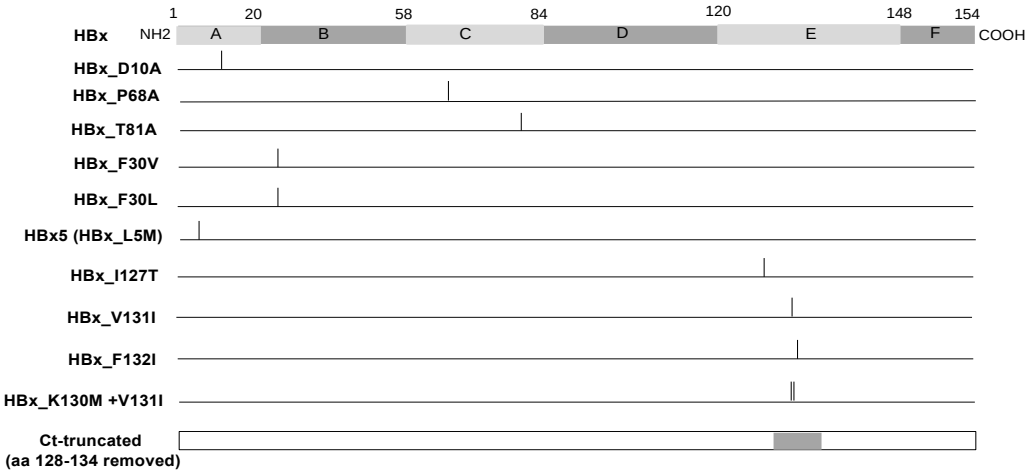

log2 FoldChanges
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
*
*
***
*
***
**
FBLIM1
NPR2
NMT1
Delta S
L-HBsAg
M-HBsAg
S-HBsAg

Figure 3. The expression of most cellular factors is not altered by individual HBV factors in HBV infection. A-D. HBx or pol do not induce expression changes in the cellular genes, while HBV capsid and L-HBsAg downregulate SRFBP1, FBLIM1, NPR2 and NMT1. HepG2-NTCP cells were transfected with plasmids that express HBx, HBc, pol or L-HBsAg. After 3 days, cells were collected for RNA isolation and qRT-PCR analysis. Data were normalized to mock and converted to log2 fold changes. **E. L-HBsAg and S-HBsAg cause the downregulation of FBLIM1, NPR2 and NMT1, while M-HBsAg is incapable of downregulating these genes.** The expression of three host genes affected by different surface antigens was quantified by qRT-PCR. In all panels, data were normalized to mock or vehicle control and converted to log2 fold changes. Results are shown as medium ± interquartile (IQR) with three independent experiments. The significant differences between the transfected samples and the mock control were analyzed by one – sample t-test and shown with asterisks "*", above the relative box (A-D). The significant differences between the delta S and samples expressing L, M, S-HBsAg were analyzed by One-Way ANOVA and shown with asterisks "*" above the relative box (E). "ns" = not significant, which is not shown in the figures. "***" = p≤0.001; "**" = p≤0.01; "*" =p ≤0.05.

A


log2 FoldChanges
4
3
2
1
0
-1
-2
-3
-4
-5
*
***
*
EXOSC9
HOMER2
HSF1
INO80C
NFYC
RFFL
MED30
SSTR5
TP53
FLG2
SMARCA4
BAD
CAB39L

B


1
20
58
84
120
148
154
HBx
NH2
A
B
C
D
E
F
COOH
HBx_D10A
HBx_P68A
HBx_T81A
HBx_F30V
HBx_F30L
HBx5 (HBx_L5M)
HBx_I127T
HBx_V131I
HBx_F132I
HBx_K130M +V131I
Ct-truncated
(aa 128-134 removed)

C

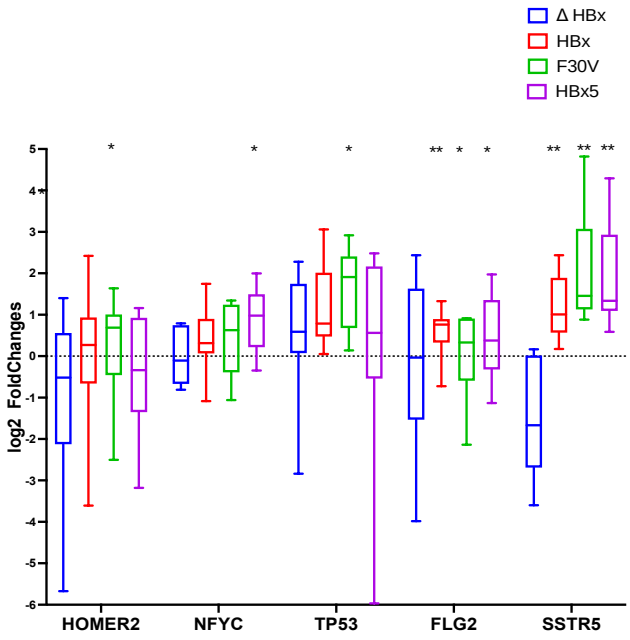

Δ HBx
HBx
F30V
HBx5
log2 FoldChanges
HOMER2
NFYC
TP53
FLG2
SSTR5

Figure 4. HBx mutants are associated with the alteration of HCC-related gene expression. A. HBx wild-type alters few HCC-related gene expressions. HepG2-NTCP cells were transfected with HBx and at 3 days post-transfection, cells were collected for RNA isolation and qRT-PCR**. B. schematic presentation of HBx wild-type and mutants.** HBx protein is dissected into six functional domains based on the homology between different mammalian hepadnaviruses. Shown at domain A, C, E are domains with more conserved residues. Also shown at domain B, D, F are domains with more variable residues. Below the domain structure of HBx is the list of HBx mutants used to test their effects on selected HCC-related gene expression. **C. HBx_F30V mutant upregulates HOMMER2, TP53, FLG2 and SSTR5, while HBx5 mutant upregulates NFYC, FLG2 and SSTR5.** The expression of selected HCC-related genes affected by HBx WT, F30V or HBx5 was analyzed using qRT-PCR**.** In all panels, data were normalized to mock control and converted to log2 fold changes. Results are shown as medium ± IQR with three independent experiments. The significant differences between the samples expressing wild type HBx and the mock (A) were analyzed by one – sample t-test and shown with asterisks "*", above the relative box. The significant differences between the delta X and samples transfected with HBx WT and mutants (C) were analyzed by One-Way ANOVA and shown with asterisks "*" above the relative box. "ns" = not significant, which is not shown in the figures. "***" = p≤0.001; "**" = p≤0.01; "*" =p ≤0.05.

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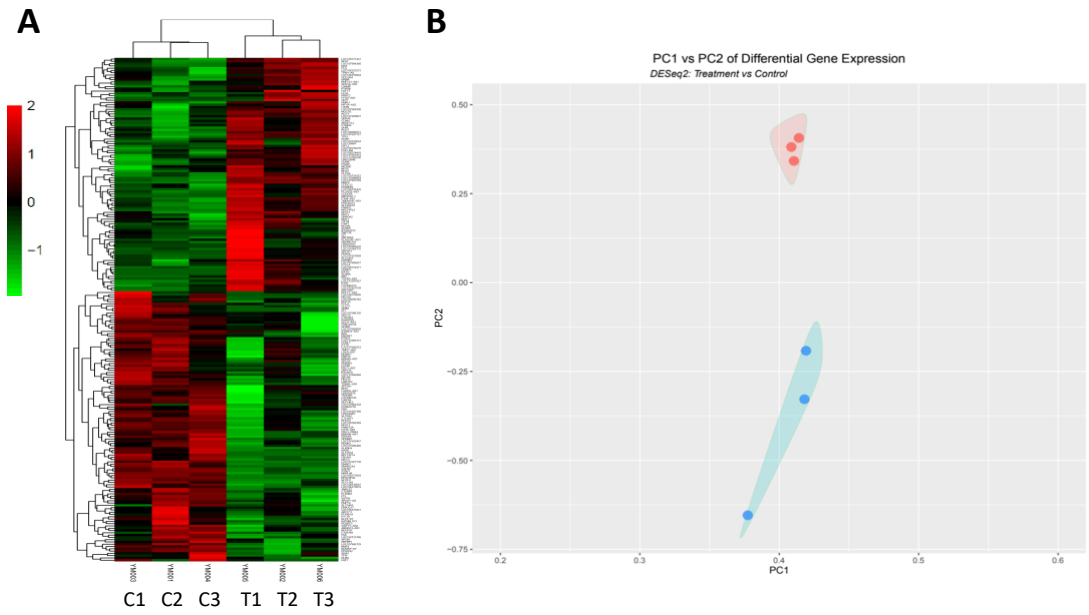
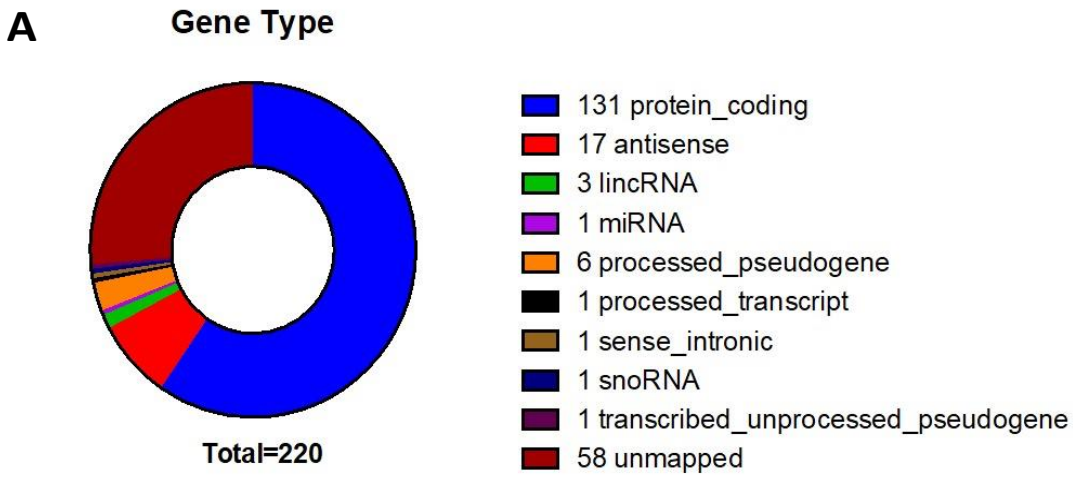
Appendices

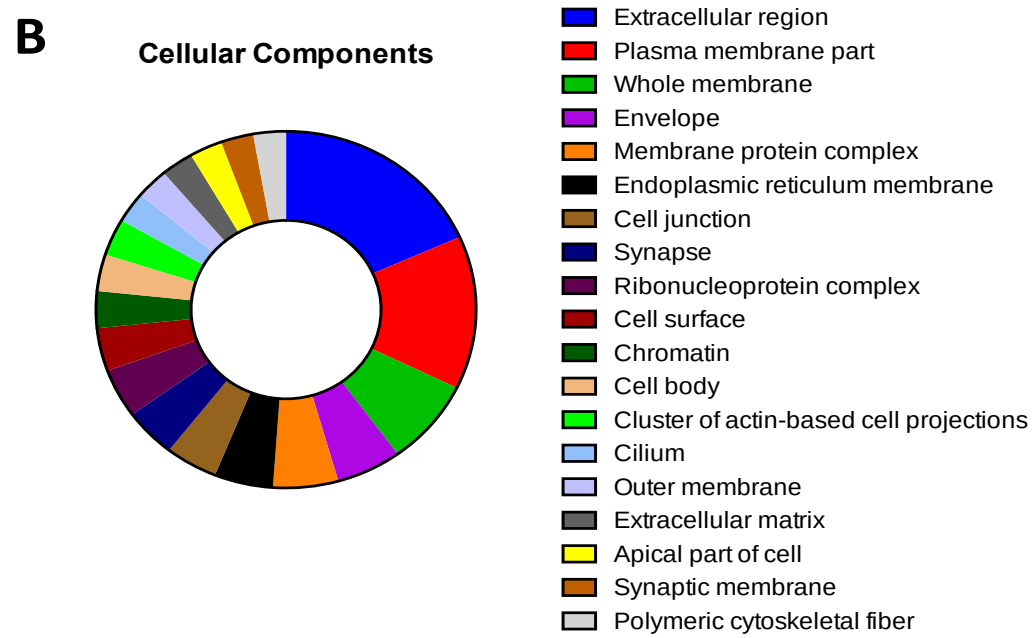


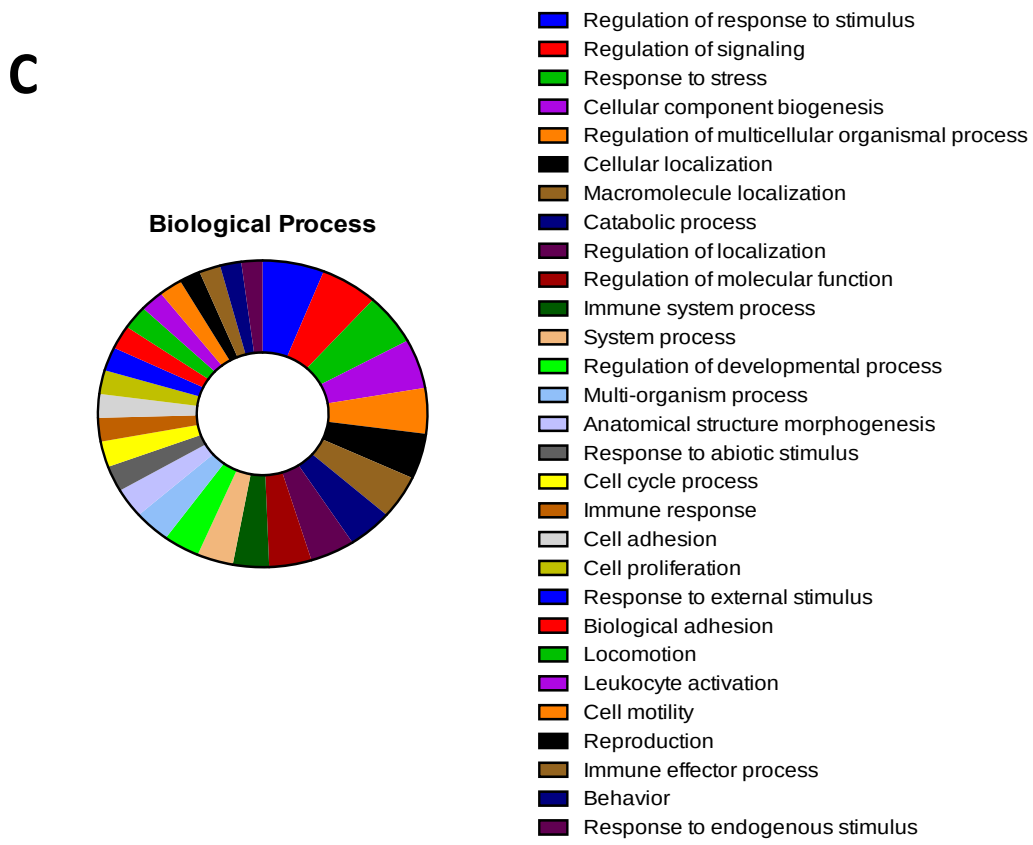
Appendix Figure 1: **Expression of 220 genes are significantly altered in HBV-infected cells. A. Heat map for the 220 significantly expressed genes.** A heat map was generated to show the changes in expression. Genes are shown on the right column and samples are indicated. The tree at the top of the figure indicates the split between control and treated samples. The tree on the left indicates the gene family groupings for the 220 genes included. C 1/2/3 represent control samples; T 1/2/3 represent treated/infected samples. **B**. **Principal component analysis (PCA) of 220 significantly altered genes in control and treated samples**. PCA shows the control (Blue) and treated (Red) samples are from two distinct clusters, indicating that there are significant differences between the groups. This analysis used the 220 gene dataset but does not use any assumptions regarding changes in gene expression.

A

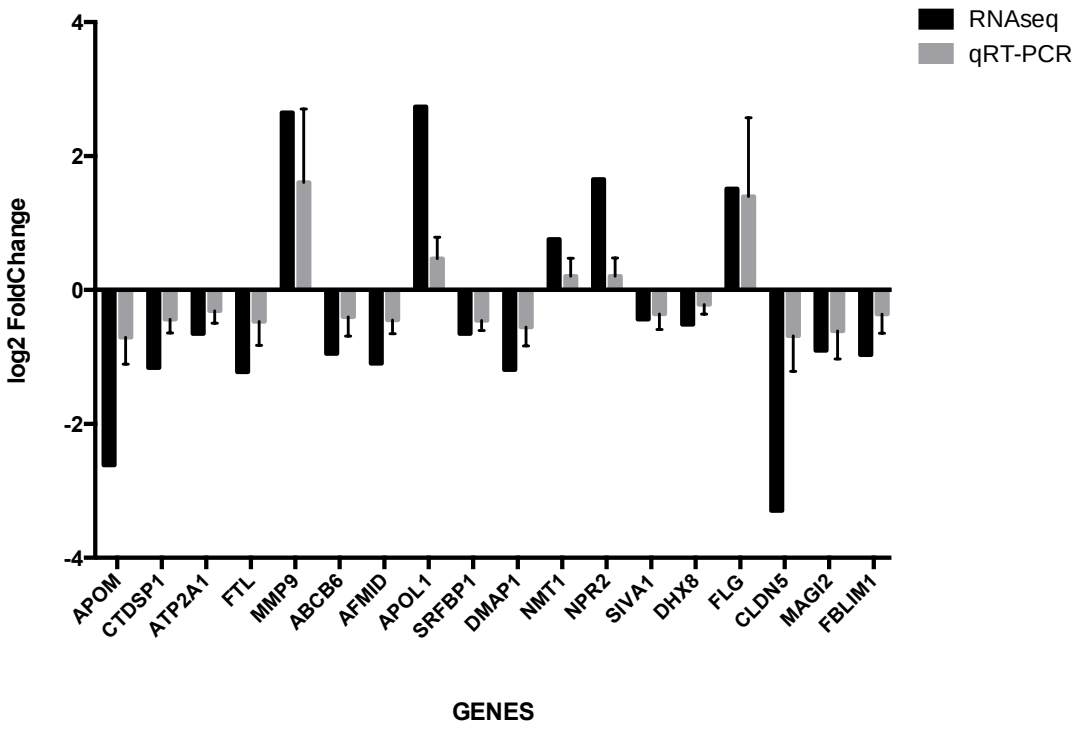

Gene Type
131 protein_coding
17 antisense
3 lincRNA
1 miRNA
6 processed_pseudogene
1 processed_transcript
1 sense_intronic
1 snoRNA
1 transcribed_unprocessed_pseudogene
58 unmapped
Total=220

B

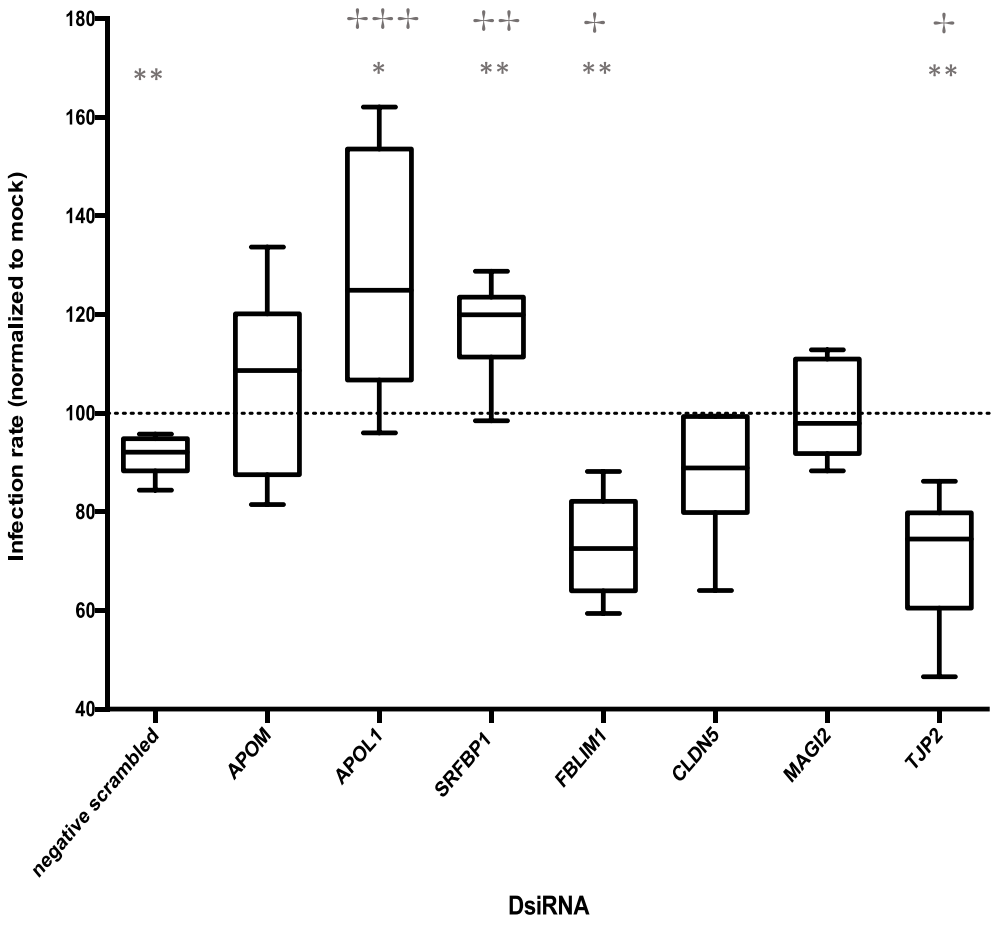

Cellular Components
Extracellular region
Plasma membrane part
Whole membrane
Envelope
Membrane protein complex
Endoplasmic reticulum membrane
Cell junction
Synapse
Ribonucleoprotein complex
Cell surface
Chromatin
Cell body
Cluster of actin-based cell projections
Cilium
Outer membrane
Extracellular matrix
Apical part of cell
Synaptic membrane
Polymeric cytoskeletal fiber



Appendix Figure 2: Gene Ontology Enrichment Analysis of genes having altered expression in HBV infected cells using ShinyGO v0.61. A. Gene type analysis of 220 significantly expressed genes. Shown is the categorization of genes based upon gene type. **B. Cellular components analysis of 131 significantly expressed protein-encoding genes.** Shown is the categorization of protein-encoding genes. **C. Biological process analysis of 131 significantly expressed protein-coding genes.** Shown is the categorization of genes based upon their association with a biological process.



Appendix Figure 3: Alteration of host gene expression was confirmed by qRT-PCR analysis. Cells were collected at 3 days post infection for RNA extraction and qRT-PCR. Results are shown as the average ± standard deviation of three independent experiments, which are compared to the RNAseq data.


Infection rate (normalized to mock)
180
160
140
120
100
80
60
40
**
+++
*
++
**
+
**
+
**
negative scrambled
APOM
APOL1
SRFBP1
FBLIM1
CLDN5
MAGI2
TJP2
DsiRNA

Appendix Figure 4: HBV infection is elevated with the knocking down of APOL1 and SRFBP1 genes, while decreased with the knocking down of TJP2 and FBLIM1 genes. Twenty-four hours post DsiRNA transfection, HepG2-NTCP cells were infected with HBV having an infection rate from 12 to 25%. Cells were collected at 5 days post infection for HBc and Alexa Fluor 594 secondary antibody staining, and flow cytometry analysis. Results are shown as the scattered dot plot with mean of six independent experiments. The significant difference between DsiRNA transfected samples and mock control was analyzed by a one sample t test, which is indicated by an "*" above the relative dot plot. The significant difference between other DsiRNA and negative scrambled DsiRNA treatment was analyzed by one-way ANOVA test, which shown with "+" above the relative dot plot. "***"/"+++"= p≤0.001; "**"/"++"= p≤0.01; "*"/"+"= p≤0.05.

Appendix Table 1: Table of gene type of 220 significantly expressed genes.

Gene Type	Gene Symbol
protein_coding	C1orf159, TARDBP, FHAD1, FBLIM1, NFYC, DMAP1, SLC35D1, CLCA4, DBT, CLCC1, HRNR, FLG, FLG2, EIF2D, B3GALNT2, ADCY3, HAAO, CALM2, PEX13, GPR45, TFPI, CTDSP1, ABCB6, FANCD2OS, GHRL, SUSD5, GOLGA4, CCDC12, HEMK1, LSAMP, CCNL1, LCORL, GNPDA2, EXOSC9, SLC9A3, EGFLAM, MOCS2, CCNH, SRFBP1, NOP16, FAM153A, APOM, GPANK1, LGSN, NT5E, KIAA0895, STX1A, GNB2, ALKBH4, CYREN, MCPH1, FDFT1, MED30, HSF1, ACO1, NPR2, FRMD3, ANGPTL2, DBH, DIPK1B, SLC34A3, NODAL, MICU1, SHLD2, FAS, LMNTD2, C11orf21, BAD, RCE1, MRPL48, CCDC65, SLC11A2, SCN8A, ZNF385A, SLC26A10, PTPRB, VEZT, CIT, HNF1A, DDX55, ANKLE2, CAB39L, DNAL1, SIVA1, FSIP1, DNAJC17, RFX7, SLC24A1, HOMER2, POLG, NME4, RHOT2, SSTR5, DNASE1, PDXDC1, ATP2A1, MAZ, PRRT2, ABCC11, KATNB1, ZNF276, TP53, IFT20, RFFL, DHX8, NMT1, AFMID, SGSH, ENOSF1, INO80C, NDUFS7, BTBD2, PLEKHJ1, SMARCA4, CEBPG, YIF1B, C19orf54, PRR19, IGFL2, FTL, ZNF776, MGME1, ID1, MMP9, SYNJ1, SEC14L2, APOL1, CDSN, PRICKLE3, PLXNB3, DKC1, PEG13
antisense	TGFB2-AS1, LINC02576, SCN1A-AS1, ITIH4-AS1, PLCXD2-AS1, WDFY3-AS2, STARD4-AS1, TONSL-AS1, APOA1-AS, TMPO-AS1, FUT8-AS1, TPM1-AS, PRC1-AS1, TMEM147-AS1, PLA2G4C-AS1, CARD8-AS1, FTCD-AS1
lincRNA	LINC02067, ARRDC3-AS1, HIF1A-AS2
miRNA	MIR4520-1
processed_pseudogene	RPL12P14, RPL21P23, PPIAP42, ARF4P3, COX6CP16, KATNBL1P3
processed_transcript	SGO1-AS1
sense_intronic	PEG13
snoRNA	SNORD110
transcribed_unprocessed_pseudogene	ZNF788P

Appendix Table 2: Summary of gene functions based on current literature.[a]

Category	No.	Genes	Alternation	Key Words	Reference
HBV associated	1	APOM	down	HBV induced regulation	PMID: 21875437, PMID: 31393946
	2	CTDSP1	down	HBV restriction	PMID: 25342750, PMID: 32265934
	3	ATP2A1	down	HBV, paramyxovirus, virus-supportive	PMID: 10871838, PMID: 30814986,
	4	FTL	down	Cellular attachment and entry of hepatitis B virus	PMID: 22359459
	5	MMP9	up	Facilitate HBV replication, upregulation by HBV; extracellular matrix organization	PMID: 28122987, PMID: 12464265
	6	CCDC12	up	HBV integration	PMID: 31417659
	7	CEBPG	up	HBx, proliferation and migration of hepatoma cells	PMID: 31621133
	8	EGFLAM	up	potential role in HBV induced **non-hodgkin lymphoma**; extracellular matrix protein	PMID: 32350851
	9	FAS	down	HBV enhances the sensitivity of hepatocytes to fas-mediated apoptosis	PMID: 30171166
	10	GHRL	up	HBV-related liver cirrhosis	PMID: 26599409
	11	ID1	down	degradation of HBx, **HCC**	PMID: 31146743, PMID: 18674781, PMID: 20574154
	12	TARDBP	down	HBV replication	PMID: 31186504
	13	HNF1A	down	Hepatocyte nuclear factor 1α downregulates HBV gene expression and replication by activating the NF-κB signaling pathway	PMID: 28319127
	14	DNASE1	down	cellular restriction factor of hepatitis B virus	PMID: 30936483
Other Viruses Associated	15	ABCB6	down	HCV-related HCC	PMID: 23483087
	16	AFMID	down	Influenza virus related downregulation, HCC	PMID: 31461941, PMID: 29449409
	17	APOL1	up	HIV-1 related, anti-viral restriction factor	PMID: 27599995
	18	SRFBP1	down	HCV entry	PMID: 26212323
	19	DMAP1	down	Antiviral defense, immune evasion and oncogenesis	PMID: 29438328
	20	NMT1	up	Elevated in DENV replication, involved in picornavirus life cycle, HIV-1 production	PMID: 30848105, PMID: 30080883, PMID: 18248763, PMID: 18089753
	21	NPR2	up	Essential for influenza virus	PMID: 23805279
	22	SIVA1	down	Required for replication of influenza A virus	PMID: 21048035
	23	DHX8	down	HIV-1 replication	PMID: 22404213
	24	FLG	up	HSV-1 replication, HPV-related cancers	PMID: 23657503, PMID: 24905740
	25	ACO1	up	COVID-19 related up-regulation	PMID: 33437935
	26	ADCY3	down	ZIKA pathogenicity	PMID: 30222212
	27	ANKLE2	down	Zika virus targeted	PMID: 31735666
	28	BTBD2	down	restrict HIV-1 infection	PMID: 21092135
	29	CCNH	up	HCV induced upregulation	PMID: 27742031
	30	DDX55	up	IAV life cycle	PMID: 27653209
	31	DKC1	up	HCC, influenza virus infection	PMID: 22912812, PMID: 24955349
	32	DNAL1	up	HIV-1 restriction	PMID: 22018492
	33	FDFT1	up	regulates HCV propagation	PMID: 24690320
	34	PEX13	down	HIV-1, HCMV, HSV-1 and ZIKA virus; autophagy	PMID: 28594894, PMID: 32127461, PMID: 30269970, PMID: 31311201, PMID: 27827795
	35	RFX7	up	HSV-1	PMID: 29114028
	36	SEC14L2	down	promote HCV replication	PMID: 26266980, PMID: 30472319
	37	SLC35D1	down	cell survival upon VSV infection	PMID: 31320712
Cell-to-cell Adhesion	38	CLDN5	down	Tight junction protein	ShinyGO v0.61: Gene Ontology Enrichment Analysis
	39	MAGI2	down	Tight junction protein	ShinyGO v0.61: Gene Ontology Enrichment Analysis
	40	FBLIM1	down	Cell-cell adhesion, cell junction organization	ShinyGO v0.61: Gene Ontology Enrichment Analysis
	41	VEZT	up	cell-cell adhesion; tumor suppressor	ShinyGO v0.61: Gene Ontology Enrichment Analysis, PMID: 24069310
	42	PRICKLE3	up	cell polarity protein, scaffold/adaptor protein	ShinyGO v0.61: Gene Ontology Enrichment Analysis
HCC or Cancer Associated	43	EXOSC9	down	its depletion attenuates tumorigenicity	PMID: 32518284
	44	HOMER2	up	HCC	PMID: 29556326
	45	HSF1	down	HCC progression, highly expressed in HBV infected patients	PMID: 25199534, PMID: 30214576
	46	INO80C	up	tumor suppressor	PMID: 28954733
	47	NFYC	up	HCC	PMID: 29774579
	48	RFFL	down	highly upregulated in HCC-R tumor tissues	PMID: 24377043
	49	MED30	down	HBV integration in tumor cells	PMID: 31200735
	50	SSTR5	down	HBV-related HCC	PMID: 31638225, PMID: 24137418
	51	TP53	up	HCC	PMID: 21760996
	52	FLG2	up	HBV-related HCC	PMID: 32017470
	53	BAD	down	HCC	PMID: 16807152
	54	CAB39L	down	tumor suppressor	PMID: 30054562
	55	SMARCA4	down	HCC	PMID: 29352111
	56	NT5E	up	cancer and immunosuppressive effects	PMID: 25298403, PMID: 29720980
	57	TFPI	down	HCC	PMID: 28053577

[a] Genes associated in both virus infection and cancer development are bolded. Genes analyzed in this study are shown in with a white box; those not analyzed are indicated with a grey box.